

Sp1 site is crucial for the mouse claudin-19 gene expression in the kidney cells[☆]

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Abstract Members of the claudin family play important roles in the formation of tight junctions in the kidneys, liver and intestine. Claudin-19 (*Cldn19*), a newly identified member of this family, is highly expressed in the kidney of the mouse. To have a better understanding on mouse claudin-19 gene expression, a 0.9-kb DNA fragment containing the 5'-flanking region of the *Cldn19* gene was isolated. DNA sequence comparison between the mouse and human *Cldn19* promoter regions exhibited little homology. One transcription initiation site was located at 104 nucleotides upstream of the start codon (ATG) of the *Cldn19* gene. The mouse claudin-19 promoter lacked typical CAAT or GC-box. Deletion constructs of the 0.9-kb DNA fragment were generated and fused to a promoterless luciferase (Luc) reporter plasmid. Transfection studies using various kidney cell lines (MDCK, mIMCD3 and HEK293) revealed that the minimal promoter fragment resided in the –39 to –108 region, which contained a number of binding sites for transcription factors including Sp1. Site-directed mutagenesis using specific oligo probes confirmed that Sp1 was crucial for *Cldn19* transactivation in the three cell lines studied. Electromobility shift assay confirmed that the nuclear extracts of these cells bound to the Sp1 oligo derived from *Cldn19* promoter, but not to the mutated Sp1 oligo probe. Moreover, this DNA–protein complex would be recognized by Sp1 antibody, indicating specific Sp1 binding. Collectively, our data suggest that Sp1 binds to the claudin-19 promoter region and is responsible for its expression in the kidney cell lines *in vitro*. © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.

Keywords: Claudin-19; Promoter; Sp1; Sp2; Sp3; Kidney; Transfection

[☆] The nucleotide sequence(s) reported in this paper is deposited in the EMBL/GenBank databases under Accession No. AF486651.

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Abbreviations: MDCK, Madin–Darby canine kidney epithelial cells; mIMCD3, murine inner renal medullary collecting duct cells; HEK293, human embryonic kidney 293

1. Introduction

The tight junction (TJ) is a specialized membrane domain at the most apical region of polarized epithelial and endothelial cells that not only creates a primary barrier to prevent paracellular transport of solutes (barrier function) but also restricts the lateral diffusion of membrane lipids and proteins to maintain the cellular polarity (fence function) [1]. More than 40 different proteins have been located to the TJs of epithelia, endothelia and myelinated cells. To date two components of the TJ filaments have been identified: occludin and claudin. The word claudin was derived from the Latin word ‘claudere’ (to close), reflecting their association with TJs. The claudin protein family comprises more than 20 members and all claudins encode 20–27 kDa proteins with four transmembrane domains [1–3].

Members of the claudin family are involved in various biophysiological processes [4] such as regulation of paracellular permeability [5,6] and conductance [7]. Although a decreased expression of claudin has been related to a number of breast tumors as well as cancer cell lines [8], a recent study did not support the involvement of aberrant claudin gene expression in the sporadic tumors and hereditary breast cancer patients. Other regulatory or epigenetic factors may be involved in the down-regulation of this gene during breast cancer development [9].

In this study, we isolated the promoter of a new member of claudin gene family, claudin-19 (*Cldn19*), from mouse testis. Mouse claudin-19 is located on chromosome 4 D2.1 containing four exons, while *Cldn2* was found in the X chromosome. To functionally characterize the promoter region of claudin-19, chimeric promoter constructs were generated and transiently transfected into mammalian kidney cell lines to identify the minimal promoter region. Furthermore, electromobility shift assay (EMSA) was performed to confirm the transcriptional factor involved in the regulation of claudin-19 expression.

2. Materials and methods

2.1. Plasmid, bacteria and reagents

pGL3-Basic, pGL3-Control and pRL-TK reporter vectors, and Dual-luciferase reporter assay system were obtained from Promega (Madison, WI, USA). DH5 α competent cells were prepared as described [10]. Restriction endonucleases, T4 DNA ligase, calf intestinal alkaline phosphatase and T4 polynucleotide kinase were purchased

Table 1
Primers used for PCR amplification of the wild type or mutated claudin-19 promoter fragment

Primer	Sequence	Site (nt)
Cldn19-1	5'-AGTTACTGTGGGTCCCTCTCT-3'	-797 to -776
Cldn19-4	5'-CAACAAAGCCTGGTTCCATAC-3'	-568 to -546
Cldn19-5	5'-CAGAGCACTGGAATCACTCCTA-3'	-296 to -274
Cldn19-6	5'-CCTGCATTCTGGAATCAGCAGC-3'	-75 to -54
Cldn19-R	5'-ATGGCCAGGTAGGAGTCT-3'	88 to 106
Unknown AP2 NF-E Sp1		
Mut 1	5'-AAAGATAGCTGCTGGGGA CTGTCTGTGGGCGG-3'	-138 to -135
Mut 2	5'-AAAGAGCTCTGCTGGGACTGTCTGTGGGCGG-3'	-138 to -135
Mut 3	5'-AAAGAGCTCTGCTGGGGA CGTGTCTGTGGGCGG-3'	-138 to -135
Mut 4	5'-AAAGAGCTCTGCTGGGGA CTGTCTGTGGGCGG-3'	-138 to -135

The putative transcriptional factor binding sites are underlined and labelled on the top. Mutated sequences are in bold.

from New England BioLabs (Beverly, MA) and Amersham BioSciences (Piscataway, NJ). Bradford reagent was from Bio-Rad (Hercules, CA). Other reagents were from Sigma (St. Louis, MO).

2.2. Isolation of 5'-flanking region of the claudin-19 gene

The promoter region of the claudin-19 was amplified using the PCR primers specific to the 5'-flanking region of the mouse *Cldn19* gene (Table 1) and *pfu* DNA polymerase (Stratagene, La Jolla, CA). In brief, the PCRs were carried out at 94 °C for 25 s and then 30 cycles of 94 °C for 25 s, 60 °C for 1 min, and 72 °C for 4 min and a final extension step at 67 °C for 4 min in a final volume of 50 µl with 10 pmol of a gene-specific primer. The PCR products were gel-purified and subcloned into pGL3-Basic vector for transfection study. Both strands of the cloned fragments were sequenced using the ABI Prism 3100 Genetic Analyser (ABI, Foster City, CA). The promoter fragment was analyzed using on-line Promoter Scan (<http://bimas.dcrf.nih.gov/molbio/proscan/>), Signal Scan (<http://bimas.dcrf.nih.gov/molbio/signal/>) and ClustalW (<http://www.ebi.ac.uk/clustalw/>) programs [11].

2.3. Construction of deletion and mutated constructs

The *XhoI/HindIII* fragment of PCR amplified DNA sequences of the claudin-19 promoter region (corresponding to region -797, -568, -296, -233, -168, -139, -108, -75 to +106) was subcloned into the *XhoI/HindIII* digested pGL3-basic vector. To create mutant claudin-19 promoter constructs, primers with nucleotides substitution were synthesized (the mutated sequences were underlined). Wild-type: 5'-AAAGAGCTCTGCTGGGACTGTCTGTGGGCGG-3'; Mut 1: 5'-AAAGATAGCTGCTGGGACTGTCTGTGGGCGG-3'; Mut 2: 5'-AAAGAGCTCTGCTGGGACTGTCTGTGGGCGG-3'; Mut 3: 5'-AAAGAGCTCTGCTGGGACTGTCTGTGGGCGG-3'; Mut 4: 5'-AAAGAGCTCTGCTGGGACTGTCTGTGGGCGG-3'.

2.4. Cell culture and transfection

The Madin-Darby canine kidney epithelial cells (MDCK), murine inner renal medullary collecting duct cells (mIMCD3) and human embryonic kidney 293 (HEK293) cells were obtained from the American Type Culture Collection (ATCC, MD, USA) and cultured in minimal essential medium (MEM), Dulbecco's minimal essential medium/Ham's F12 (DMEM/F12) and DMEM, respectively (Gibco-BRL, Gaithersburg, MD), supplemented with 10% fetal bovine serum, penicillin (50 U/ml), streptomycin (50 U/ml) and 2 mM L-Glutamine at 37 °C and 5% CO₂. Cell transfection experiments were carried out using Fu-

Gene 6 transfection reagent (Roche Molec. Biochem., Germany) as described previously [12]. Briefly, the cells were grown on a 24-well tissue culture plate so that the cell layer was 70% confluent on the day of experiment. For each well, 500 µl of medium containing 1.5 µl of FuGene 6 was mixed with 500 ng of test plasmid and 50 ng of pRL-TK plasmid and incubated for 30 min at room temperature. FuGene6-DNA complex was added slowly to each dish and the dish was incubated at 37 °C for 48 h. The luciferase activity was quantified using the Dual-luciferase reporter assay system (Promega) and a luminometer in accordance with the manufacturer's protocols. All the experiments were repeated thrice in duplicates.

2.5. Electromobility shift assay

Nuclear protein extracts from MDCK, mIMCD3 and HEK293 cells were prepared using the NucBuster Protein Extraction kit (Novagen, Madison, WI) and quantified using the Bradford protein assay kit (Bio-Rad, Hercules, CA). The double-strand oligo probes (Table 2) were labelled with [γ -³²P]ATP (Amersham BioSciences) using the Ready-To-Go T4 Polynucleotide kinase kit (Amersham BioSciences). Unincorporated nucleotides were removed by spin column in the TE buffer. Ten microgram of nuclear extract was incubated with or without unlabelled oligo probes or Sp1 (PEP2, Santa Cruz Biotech. Inc., Santa Cruz, CA) antibody in 1× binding buffer (4% glycerol, 1 µg poly(dI-dC)·(dI-dC), 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA and 0.5 mM DTT) in a 10 µl reaction for 10 min at room temperature. Then, radiolabelled probe was added and incubated for further 30 min at room temperature. The reaction products were analyzed on a 6% non-denaturing polyacrylamide gel. The gels were dried under vacuum and autoradiographed on Kodak MS films overnight at -70 °C.

2.6. Statistical analysis

All results are expressed as means ± S.E.M. Statistical comparisons were performed with a paired Student's *t* test.

3. Results

3.1. Cloning of the *Cldn19* promoter

The putative promoter region of the mouse claudin-19 gene was obtained by PCR amplification using the primers specific to the 5'-flanking region of the claudin-19 cDNA sequence. When compared with the cDNA sequence from the GenBank database, the putative mouse claudin-19 promoter region was localized. The fragments were gel-purified and cloned into the pGL3-basic vector. Putative transcription factor binding sites of the longest amplified fragment were identified (Fig. 1). However, no conserved CAAT and TATA-box sequences were found. Sequence comparison of the 5'-flanking region of the mouse claudin-19 promoter with the human counterpart using

Table 2
Primers used for EMSAs

Primer	Sequence
Cldn19-cSp1 (consensus)	5'-ACTGTCTGTGGGCGGGTTTGG-3'
Cldn19-mSp1 (mutant)	5'-ACTGTCTGTGGGCGGGTTTGG-3'

The putative Sp1 site is marked with bold, mutated nucleotides are underlined.

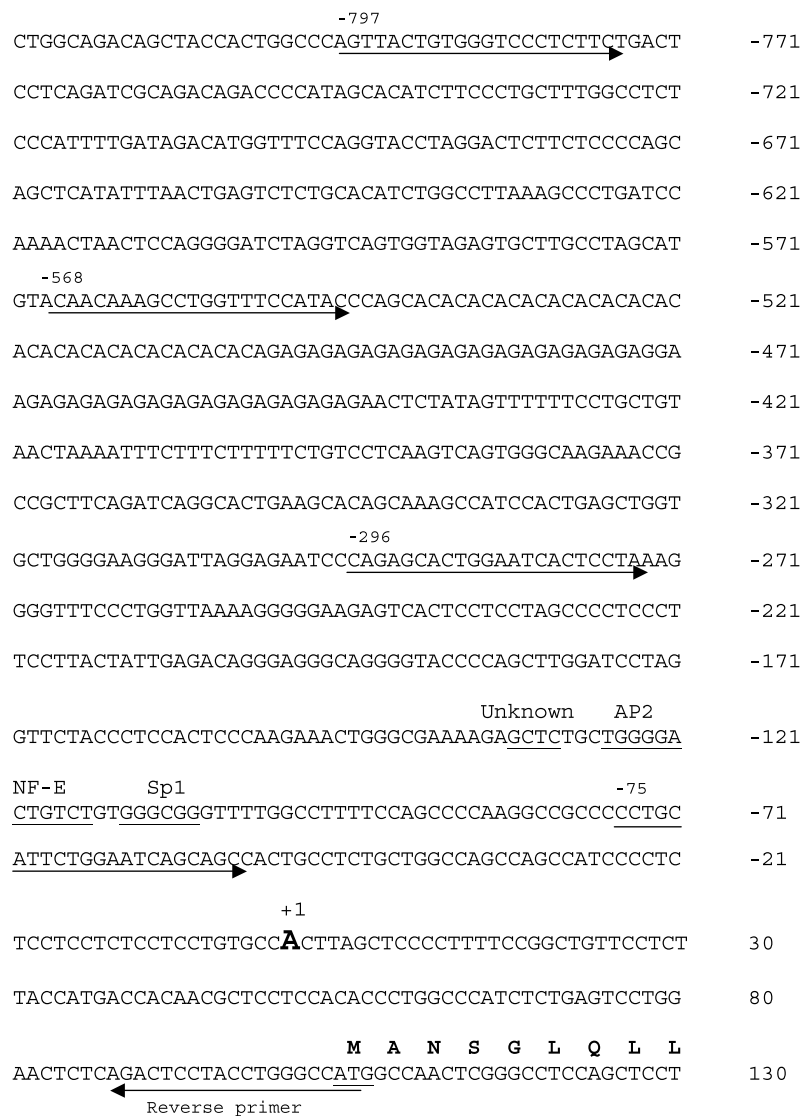


Fig. 1. The nucleotide sequence and putative regulatory elements of the 5'-flanking of mouse *claudin-19* gene. The large capital A residue at position +1 indicates the transcriptional initiation site as determined by RACE method. The translation start site (ATG) is underlined. The encoding amino acid sequence of the first exon of *Cldn19* is indicated by bold and capital letters on the top. The putative transcriptional factor binding sites (unknown, AP2, NF-E and Sp1) located between -139 and -75 are shown. The primer sequences used for construction of claudin-19 deletion constructs are marked with arrows.

the GCG SeqWeb program did not show any significant homology (data not shown). The transcription start site of claudin-19 was determined by 5'-RACE method (data not shown). The longest PCR product obtained corresponded to the unique transcription start site of 104 nucleotides ahead of the translation start site of the claudin-19 gene (Fig. 1).

3.2. Transfection of *Cldn19* promoter into kidney cell lines

Chimeric constructs containing various lengths of the *Cldn19* promoter were tested in three kidney derived cell lines: MDCK, mIMCD3 and HEK293 (Fig. 2). The luciferase activities of pGL-797/106Luc (0.8-kb), pGL-568/106Luc (0.6-kb) and pGL-296/106Luc (0.4-kb) in all the three cell lines were significantly increased (4–7-fold increase) when compared to the pGL-75/106Luc (0.2-kb) or the empty construct pGL3-Basic (Fig. 2).

3.3. Determination of *Cldn19* minimal promoter region

To localize the minimal promoter region of the claudin-19 gene, four additional deletion constructs (pGL-223/106Luc, pGL-168/106Luc, pGL-139/106Luc and pGL-108/106Luc) were generated specially between the -296 and -75 regions (Fig. 3). Transfection of these constructs into the MDCK cell line demonstrated that pGL-139/106Luc retained strong promoter activity. Further deletion (pGL-108/106Luc) resulted in a drastic decrease in promoter activity, suggesting that the functional element of the claudin-19 promoter resided between the -139 and -108 region. Interestingly, the inclusion of the -168 to -139 regions suppressed promoter activity ($P < 0.05$), suggesting the presence of a silencer element in this region. Detailed sequence analysis of the mouse claudin-19 promoter region using Signal Scan program (<http://bimas.dcrn.nih.gov/molbio/signal/>) revealed one unknown and three

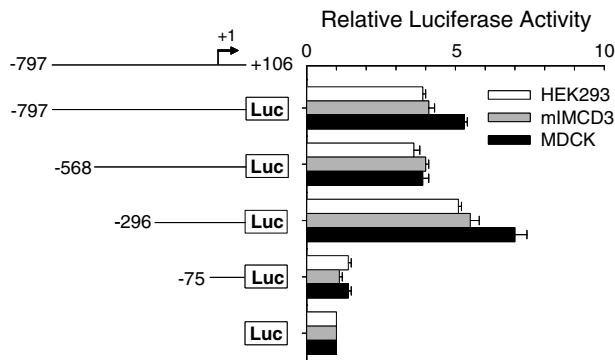


Fig. 2. The transactivation of luciferase reporter constructs by *Cldn19* promoter in MDCK, mIMCD3 and HEK293 cells. Various claudin-19 promoter-Luc chimeric constructs are transiently transfected into the cells. The Luc expression of the empty expression vector is taken to be 1 and relative activities of each *Cldn19* promoter are normalized with pRL-TK expression levels. Values are means \pm S.E.M. of at least three independent experiments.

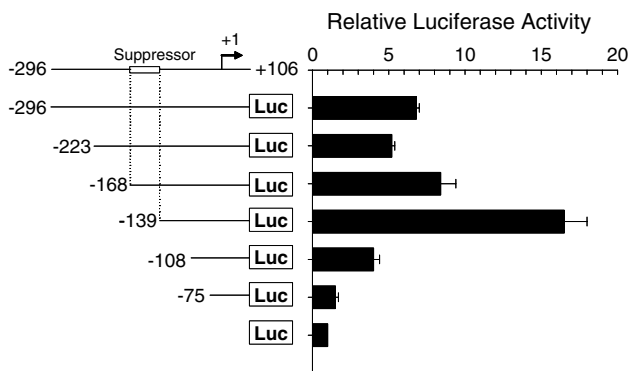


Fig. 3. The transactivation of luciferase reporter constructs by *Cldn19* promoter in MDCK cells. Various claudin-19 promoter-Luc chimeric constructs (pGL-296/106Luc, pGL-223/106Luc, pGL-168/106Luc, pGL-139/106Luc, pGL-108/106Luc and pGL-75/106Luc) are transiently transfected into the cells. The Luc expression of the empty expression vector (pGL-3Basic) is taken to be 1 and relative activities were normalized with pRL-TK expression levels. Values are means \pm S.E.M. of three independent experiments.

transcriptional factor-binding sites for AP2, NF-E and Sp1 at this region (Fig. 1).

3.4. Site-directed mutagenesis of the *Cldn19* promoter

To study the role of these transcriptional factors on the mouse claudin-19 promoter activity, mutant constructs specific to these transcriptional factor-binding sites were generated by site-directed PCR amplification protocol. Mut1, Mut2, Mut3 and Mut4 primers were specific to the unknown, AP2, NF-E and Sp1 transcription factor binding sites, respectively. Transfection of these mutated constructs into the three cell lines demonstrated that Sp1 binding site mutations resulted in a drastic decrease in the promoter activity, suggesting that Sp1 site is crucial for claudin-19 expression in kidney cells (Fig. 4). Other mutation constructs did not show significant changes in the promoter activities.

3.5. Specific binding of Sp1 protein on the *Cldn19*-cSp1

To further demonstrate that the Sp1 protein interacts with this region, EMSA using *Cldn19*-cSp1 consensus oligo and

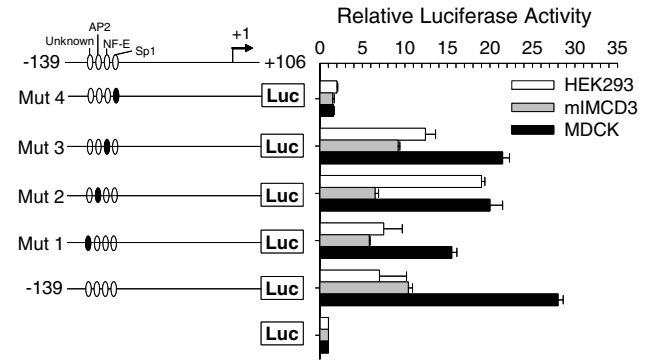


Fig. 4. The transactivation of mutant constructs on luciferase expression in MDCK, mIMCD3 and HEK293 cells. Various claudin-19 promoter-Luc mutant constructs (Mut 1, Mut 2, Mut 3 and Mut 4; see Table 1) are transiently transfected into the cells. The Luc expression of the empty expression vector (pGL-3Basic) is taken to be 1 and relative activities of the mutant constructs are normalized with pRL-TK expression levels. Values are means \pm S.E.M. of three independent experiments. Open oval: wild-type sequence, filled oval: mutant for the unknown, AP2, NF-E or Sp1 site (see Table 1).

MDCK, mIMCD3, HEK293 or HeLa cell nuclear extracts was carried out. Our result demonstrated that all the three kidney nuclear extract complexes with *Cldn19*-cSp1 oligo formed one major retarded band (Fig. 5A), but not with the *Cldn19*-mSp1 mutated oligo. Addition of 10–50-fold excess of cold and specific probe, but not non-specific probe displaced the binding of the MDCK nuclear extract (Fig. 5B). In order to confirm the specific Sp1 protein–DNA interaction, we used antibody against Sp1 protein for the supershift experiment. Addition of the Sp1 antibody resulted in a further retardation of the DNA–protein complex for both MDCK and HeLa cells, suggesting that Sp1 is present in the DNA–protein complex. It was noted that further addition of Sp1 antibody might destabilize the DNA complex in the EMSA experiment (Fig. 5B, lane 8). Neither Sp2 nor Sp3 antibody could bind the DNA–protein complex in the EMSA study (data not shown).

4. Discussion

Members of the claudin family play important roles in the formation of TJs in the kidneys, liver, brain and intestine [1–3,13–15]. In this study, the promoter region of claudin-19 (*Cldn19*), a newly identified member of this family, was isolated and characterized by transfection and EMSA studies. In mammalian cells, gene expression is frequently controlled at the level of transcription [16]. The control region of typical eukaryotic genes comprises proximal (core) and distal (enhancer) promoter regions. The core promoter region consists predominantly of two elements: the TATA-box and/or the Inr elements (CTCANTCT at -3 to $+5$ position relative to the transcriptional start site) [17,18] which can be present either alternately (i.e., either TATA⁺Inr⁻ or TATA⁻Inr⁺) or in limited cases, simultaneously (TATA⁺Inr⁺) [19]. In the mouse *Cldn19* promoter, no typical CAAT and TATA-box were found (GenBank Accession No. NT_039265). The Inr element of *Cldn19* gene (GCCACTTA at -3 to $+5$ position, conserved residues were underlined) showed weak homology with the consensus sequence. Trans-activation of TATA⁻Inr⁺ promoter is

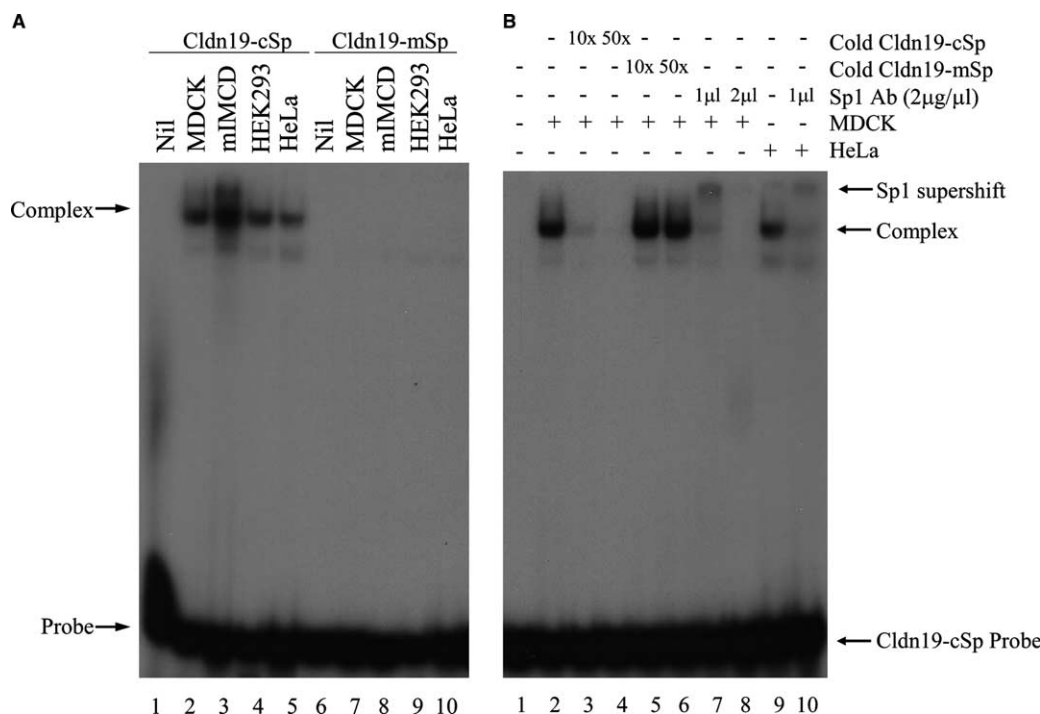


Fig. 5. EMSA using MDCK, mIMCD3, HEK293 or HeLa cell nuclear extracts on Sp1 binding. (A) Radiolabelled Cldn19-cSp1 consensus and Cldn19-mSp1 mutant oligos are incubated with MDCK, mIMCD3, HEK293 or HeLa nuclear extract (NE) and resolved on a 6% polyacrylamide gel. (B) Specific DNA–protein complex is confirmed by competition assay using 10–50-fold excessive cold Cldn19-cSp1 (consensus) or Cldn19-mSp1 (mutant) oligo probes and specific antibody raised against Sp1 protein for supershift experiment. The formation of further retarded band (supershift) at the top with the disappearance of the protein–DNA complex below confirms the specific Sp1 binding.

mediated by interaction between TFII-I and the Inr element to recruit TFIID and other components to the promoter [20].

In this study, the minimal 0.3-kb mouse *Cldn19* promoter (pGL-296/106Luc) displayed full transactivation activities. Interestingly, increase of the length of the promoter (up to 1-kb, pGL-797/106Luc) suppressed the transactivation activity, notably between the –139 and –168 region in all the three kidney cell lines tested, suggesting the presence of suppressor element in this region. Several putative transcriptional factor binding sites were found in this region, including CAP site, W-element and LBP-1 site. Whether these elements are important for the *Cldn19* gene expression needs further investigation.

By using site-directed mutagenesis and EMSA, we confirmed that Sp1 is crucial for *Cldn19* expression in the kidney cell lines studied. Higher trans-activation of the *Cldn19* promoter was found in the MDCK than in the HEK293 and mIMCD3 cells, albeit they are all kidney derived cell lines. This observation suggested that factor(s) in addition to Sp1 may co-regulate *Cldn19* promoter activity, although the identity of this factor remains obscure. This observation was supported by the fact that Sp1 expression in mouse tissues was highest in the thymus, lung and spleen than the other tissues such as kidney [21], where claudin-19 is highly expressed. In addition, it was reported that Sp1 protein expression in normal tissue varies markedly during development [21]. Sp1 mRNA levels increase in CV-1 cells in response to SV40 viral infection and in T-lymphocytes in response to phorbol ester treatment [22]. Therefore, it would be important to study the regulation of

claudin-19 expression by manipulating Sp1 expression in vitro and in vivo. Mutation of the unknown transcription factor binding site, AP2 and NF-E did not abolish the promoter activity. However, mutation of the Sp1 site almost completely abolished the promoter activity in these cell lines. EMSA showed that Sp1 protein binds to the putative Sp1 site on the *Cldn19* oligo. Addition of Sp1 but not Sp2 or Sp3 antibody specifically binds to the DNA–Sp1 complex and causes a supershift product in the gel. However, excessive Sp1 antibody destabilizes the protein–DNA product in the supershift experiment, suggesting that Sp1 antibody may perturb stable Sp1 protein–DNA complex by competing with Sp1 oligo for Sp1 binding site in vitro.

The 5'-flanking region of the human and mouse claudin-2 gene contains binding sites for intestine-specific Cdx homeodomain proteins and hepatocyte nuclear factor (HNF)-1 [23]. However, no HNF and Cdx sites were found in our putative *Cldn19* promoter region. Thus, at least for claudins-19 and -2, a differential mode of transcriptional regulation can be suggested. It is known that Sp1 interacts with a variety of gene promoters containing GC-box elements for constitutive expression of genes in different tissues [24], including hepatocyte growth factor receptor gene in the kidney [25]. Whether co-regulation of Sp1-mediated transcription occurs in kidney remains an open question.

In conclusion, the minimal promoter region of *Cldn19* was mapped to the –139 region relative to the transcription start site. An Sp1 site plays an important role in the expression of the gene in the kidney cell lines.

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