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Transcriptional Regulation of the Human TLR9 Gene

Fumihiko Takeshita, Koichi Suzuki, Shin Sasaki, Norihisa Ishii, Dennis M. Klinnman, and Ken J. Ishii

To clarify the molecular basis of human TLR9 (hTLR9) gene expression, the activity of the hTLR9 gene promoter was characterized using the human myeloma cell line RPMI 8226. Reporter gene analysis and EMSA demonstrated that hTLR9 gene transcription was regulated via four cis-acting elements, cAMP response element, 5′-PU box, 3′-PU box, and a C/EBP site, that interacted with the CREB1, Ets2, Elf1, Elk1, and C/EBPα transcription factors. Other members of the C/EBP family, such as C/EBPβ, C/EBPδ, and C/EBPε, were also important for TLR9 gene transcription. CpG DNA-mediated suppression of TLR9 gene transcription led to decreased binding of the trans-acting factors to their corresponding cis-acting elements. It appeared that suppression was mediated via c-Jun and NF-κB p65 and that cooperation among CREB1, Ets2, Elf1, Elk1, and C/EBPα culminated in maximal transcription of the TLR9 gene. These findings will help to elucidate the mechanism of TLR9 gene regulation and to provide insight into the process by which TLR9 evolved in the mammalian immune system.

GGG (23); and 1471, TCA AGC TTG A (uppercase letters indicate phosphorothioate and lowercase letters indicate phosphodiester). LPS, IFN-γ, and CQ were purchased from Sigma-Aldrich (St. Louis, MO). Anti-CREB1, activating transcription factor 2 (ATF2), -Ets1/2, -Ets2, -Elk1, -Elk1, -Sp1 (PU.1), -C/EBPα, -C/EBPβ, -C/EBPγ, -C/EBPδ, and -Sp1 Abs were from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell lines and cell cultures**

RPMM 8262 and THP-1 cells were purchased from American Type Culture Collection (Manassas, VA). Cells were grown in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS, 50 μg/ml penicillin/streptomycin, and 0.1 mM nonessential amino acids. Cells were maintained at 37°C in 5% CO₂.

**RT-PCR**

Semi-quantitative RT-PCR was performed as previously described (21). Briefly, total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol, and 5 μg of total RNA was reverse transcribed in first strand buffer (50 mM Tris-HCl (pH 7.5), 75 mM KCl, and 2.5 mM MgCl₂) containing 25 ng/μl oligo(dT)₁₂–₁₈, 200 U of Moloney leukemia virus reverse transcriptase, 2 mM dNTP, and 10 mM DTT. Reactions were incubated at 42°C for 1 h, after which 1 μl of aliquots of the cDNA synthesis mixture was subjected to standard PCR for 27 or 35 cycles using primers shown in Table I. PCR products were separated on 1.5% agarose gels and visualized under UV light after ethidium bromide staining.

**Cloning of the hTLR9 gene 5′-flanking region and construction of hTLR9 gene promoter-dependent luciferase expression vectors**

The 5′-flanking region of the hTLR9 gene (from the translation start site to 3227 bp upstream) was PCR amplified from human genomic DNA using the primers MluI–3227TLR9 S primer (5'-CGA GCG TTC GGC GGA CAG GGT GAC ACT AGG GTC CCA CCA GAG CAG CAG CAG GGC CTC CTC CAG AGG CTC-3'), HindIII–1/TLR9 AS primer (5'-CCG AAG CTT GCT GGG GGG CAG CAG CTT CTC CAG AGG CTC GTC-3'), truncation mutants of the 5′-flanking region were also PCR amplified. PCR fragments were cloned into the MluI/HindIII site of the pGL3 basic vector (Promega, Madison, WI). Two-step PCR mutagenesis was performed to obtain site-directed mutants. Mutated sequences included the CAMP response element (CRE) (R1: 68–182GTTGAC), truncated to mutation at position 239: 68–239GTTGACATA. For each mutation, a primer pair was synthesized to PCR amplify the fragment of interest, and the corresponding region was cut from human genomic DNA using the primers shown in Table I. The PCR products were gel purified and cloned into the mammalian expression vector pCMV (Promega). Sequences of the PCR products were confirmed using an ABI PRISM Genetic Analyzer (Perkin Elmer Biosystems).

**Construction of transcription factor expression vectors**

Human cDNAs encoding Ets1, Ets2, Ets1, Ets1, Sp1, Sp1, P53, CREB1, ATF2, C/EBPα, C/EBPβ, C/EBPγ, and C/EBPδ were amplified by PCR using a Marathon-Ready cDNA library derived from human spleen (BD Clontech, Palo Alto, CA) and primers as shown in Table I. Mouse cDNA encoding c-Fos, c-Jun, or NF-κB p65 were also PCR amplified from the mouse spleen cDNA library using primers shown in Table I. All PCR products were gel-purified and cloned into the mammalian expression vector pCMV (Promega). Sequences of the PCR products were confirmed using an ABI PRISM Genetic Analyzer (Perkin Elmer Biosystems).

**5′-RACE**

5′-RACE was performed on hTLR9 cDNA using a SMART RACE cDNA Amplification Kit (BD Clontech). A 36-cycle touchdown PCR, in which the annealing temperature was reduced 2°C every 12 cycles from 70°C, was performed using the AP-1 primer (BD Clontech), the +36/5TLR9 AS primer, the -5′ACC GCC AAG AAG GCT GCC ATG GAC-3′, and the Marathon-Ready cDNA library derived from human spleen (BD Clontech). A 35-cycle nested PCR was then performed using the AP2 primer (BD Clontech), HindIII–1/TLR9 AS primer, and 5 μl of aliquots of first-round PCR products. Resultant PCR products were cloned into the pGEM-T vector (Promega) and inserts of 10 independent clones were sequenced.

**Transient transfection and luciferase assay**

Transient transfections were conducted using FuGene 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s protocol. RPMI 8262 cells (1 × 10⁶) were cotransfected with Firefly luciferase vector, control Renilla luciferase vector (pTK-RL), and/or expression vectors for each transcription factor to give a constant 550 ng of DNA/reaction. Eight hours after transfection, cells were treated with medium alone, 1 μg/ml LPS, 100 Units/ml IFN-γ, or 1 μl ODN in the presence or absence of CQ for an additional 40 h. Cells were lysed in 200 μl of 1× Passive Lysis Buffer (Promega), and luciferase activity in 10 μl of aliquots of the cell lysates was measured using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocols. Firefly luciferase activity of individual cell lysates was normalized against Renilla luciferase activity or protein concentration as measured using the BCA protein assay kit (Bio-Rad, Hercules, CA).

**EMSA**

Nuclear extracts were prepared as previously described (20). All buffers used contained 1 mM Na₂VO₃ and Complete Mini protease inhibitor mixture (Roche Diagnostics). DNA probes were end-labeled with [γ-³²P]ATP

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Table I. Primers used for RT-PCR and construction of mammalian expression vectors

<table>
<thead>
<tr>
<th>Target cDNA</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>5′-CAT ACG TCT GCT GAT CAC C-3′</td>
<td>5′-TTC TAG GAA TGG ACT GCC GGC-3′</td>
</tr>
<tr>
<td>TLR2</td>
<td>5′-GAT GAC TCT ACC AGA TGC C-3′</td>
<td>5′-TCG TTA GTC TGC TGG GGT CAC-3′</td>
</tr>
<tr>
<td>TLR3</td>
<td>5′-CTC AGA AGA TCA GCA GGC-3′</td>
<td>5′-GGA ATT CGA AAT GAG CAC-3′</td>
</tr>
<tr>
<td>TLR4</td>
<td>5′-TTA CTT GTG TGA CTC TCC ATC C-3′</td>
<td>5′-TTG TAG TGA TGG TAA CAC-3′</td>
</tr>
<tr>
<td>TLR5</td>
<td>5′-CCT TGA CTA TGA AGA AAG CAG C-3′</td>
<td>5′-TCC TGA CCA AAT GCA GCC-3′</td>
</tr>
<tr>
<td>TLR6</td>
<td>5′-CTC ACT GAA GGA AAA TCC-3′</td>
<td>5′-GGA ATT CGA AAT GAG CAC-3′</td>
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<tr>
<td>TLR7</td>
<td>5′-ACC ACG ACC ACC CAC CTC ACT C-3′</td>
<td>5′-GGA ATT CGA AAT GAG CAC-3′</td>
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<tr>
<td>TLR8</td>
<td>5′-CGA ACA ATT CCA AGG CCG CAC-3′</td>
<td>5′-GGA ATT CGA AAT GAG CAC-3′</td>
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<tr>
<td>TLR9</td>
<td>5′-CAA CAT CCT CAC GTG GGC-3′</td>
<td>5′-CAG ACC GGA GAC CAA CAC-3′</td>
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<td>TLR10</td>
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<td>5′-CGT TTA GAA GCC CAT GGC-3′</td>
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<tr>
<td>RPIF05</td>
<td>5′-CTG CTT CTG GTG GGC TGC C-3′</td>
<td>5′-GAA GTC ATT AGC TCA TCC-3′</td>
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<tr>
<td>GAPDH</td>
<td>5′-ACC ACC ATG AGC AGG GGC-3′</td>
<td>5′-GAG TCA GTA GGA GAG CAC-3′</td>
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<tr>
<td>Ets1</td>
<td>5′-GCA GGG TTC GGC CAC CAC TGG CAC CAC CAT GAA-3′</td>
<td>5′-GAC TAG CCA TCT GAT GCC CAC-3′</td>
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<tr>
<td>Ets2</td>
<td>5′-GCA GGG TTC GGC CAC CAC TGG CAC CAC CAT GAA-3′</td>
<td>5′-GTC CTA GAC TCT GAC TGG GGC CAC-3′</td>
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<tr>
<td>Sp1 (PU.1)</td>
<td>5′-GCA GGG TTC GGC CAC CAC TGG CAC CAC CAT GAA-3′</td>
<td>5′-GTC CTA GAC TCT GAC TGG GGC CAC-3′</td>
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<tr>
<td>Sp8</td>
<td>5′-GCA GGG TTC GGC CAC CAC TGG CAC CAC CAT GAA-3′</td>
<td>5′-GTC CTA GAC TCT GAC TGG GGC CAC-3′</td>
</tr>
<tr>
<td>CREB1</td>
<td>5′-GCA GGG TTC GGC CAC CAC TGG CAC CAC CAT GAA-3′</td>
<td>5′-GTC CTA GAC TCT GAC TGG GGC CAC-3′</td>
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<tr>
<td>ATF2</td>
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<td>5′-GTC CTA GAC TCT GAC TGG GGC CAC-3′</td>
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<td>C/EBPα</td>
<td>5′-GGA ATT CCA GAT GAA GAA GTT TAC AGG AGG ATG-3′</td>
<td>5′-GAC TAG CCA TCT GAT GCC CAC-3′</td>
</tr>
<tr>
<td>C/EBPβ</td>
<td>5′-GGA ATT CCA GAT GAA GAA GTT TAC AGG AGG ATG-3′</td>
<td>5′-GAC TAG CCA TCT GAT GCC CAC-3′</td>
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<tr>
<td>C/EBPγ</td>
<td>5′-GGA ATT CCA GAT GAA GAA GTT TAC AGG AGG ATG-3′</td>
<td>5′-GAC TAG CCA TCT GAT GCC CAC-3′</td>
</tr>
<tr>
<td>CREB1</td>
<td>5′-GGA ATT CCA GAT GAA GAA GTT TAC AGG AGG ATG-3′</td>
<td>5′-GAC TAG CCA TCT GAT GCC CAC-3′</td>
</tr>
<tr>
<td>NF-κB</td>
<td>5′-GGG ACC AGT CTT ACC ATG AGC GAT CTG TTG CCG-3′</td>
<td>5′-GGG ACC AGT CTT ACC ATG AGC GAT CTG TTG CCG-3′</td>
</tr>
</tbody>
</table>
Characterization of TLR family gene expression in RPMI 8226 cells

To characterize the gene expression profile of TLR family genes in RPMI 8226 and THP-1, RT-PCR targeting TLR1–10, RP105, and GAPDH was performed. As shown in Fig. 1, RPMI 8226 cells constitutively expressed mRNA for TLR1, 4, 6, 7, 9, and 10 and RP105, whereas THP-1 cells expressed all TLR family members except TLR9. The TLR expression profile in RPMI 8226 cells was identical with that of primary B cells, as previously reported by Hornung et al. (18).

It has been demonstrated previously that CpG DNA reduces TLR9 mRNA expression levels in both macrophages and B cells. To confirm this finding in RPMI 8226 cells, TLR9 mRNA levels were monitored by semiquantitative RT-PCR after incubation of cells with or without 1 μM CpG DNA (K3), 1 μg/ml LPS, or 100 U/ml IFN-γ (Fig. 2). TLR9 mRNA levels significantly decreased 16 h after K3 treatment (to ~27% of original level), whereas LPS treatment decreased expression levels ~69% (Fig. 2, a and c). In contrast, IFN-γ treatment did not alter hTLR9 expression levels.

**Determination of transcription start sites of the hTLR9 gene**

To examine the molecular mechanisms underlying transcriptional regulation of the hTLR9 gene, the 5’-flanking region, from the translation start site to ~3.2 kb upstream, was cloned and 5’-RACE was performed to determine the transcription start site. Nine of 10 clones showed identical cDNA ends (referred to as the major transcription start site), and one clone defined a shorter cDNA end (referred to as the minor transcription start site). The major and minor transcription start sites were mapped to ~91 and ~79 bp from the translation start site, respectively (Fig. 3, large arrow and small arrow, respectively).

**Identification of cis-acting elements within the hTLR9 gene promoter**

As shown in Fig. 3, a number of putative binding sites for transcription factors were found within an 800-bp upstream region. To examine TLR9 gene promoter activity, a reporter gene assay was performed using truncated and mutagenized forms of the TLR9 gene promoter within 5’-flanking region-luciferase gene hybrid.
constructs, as shown in Fig. 4. RPMI 8226 cells transfected with pGL3227/H11002, which contained the region from 3227 to 700 bp relative to the translation start site, showed >15.2-fold more luciferase activity compared with cells transfected with pGL basic. This suggested that the promoter was constitutively active in RPMI 8226 cells. To identify essential cis-acting elements important for promoter activity, 5'- and/or 3'-truncated forms of the construct were tested. Although deletion of the 5'-end of the flanking region from 3227 to 700 bp did not alter promoter activity, further deletion of the 5'-end from 700 to 640 bp resulted in a ~62% reduction in luciferase activity (Fig. 4Aa). Deletion of the 5'-end from 640 to 290 bp did not affect luciferase activity, but deletion between 290 to 240 bp resulted in decreased activity to ~53% (Fig. 4Ab). Deletion between ~160 and ~130 bp also resulted in an ~40% decrease in activity (Fig. 4Ac). Deletion of the 3'-end of the flanking region from 1 to 68 bp did not affect luciferase activity, whereas further deletion from 68 to 120 bp resulted in an ~80% decrease in activity (Fig. 4Ad). These results suggested that there were at least four regions that critically regulated promoter activity (between 700 and 640 bp, between 290 and 240 bp, between 160 and 130 bp, and between 120 and 68 bp). A typical TATA box was found in the 160 to 130 bp region, which suggested that this region was required for basic transcriptional initiation. Consensus motifs recognized by known transcription factors were identified within the four regions, including CRE (~686 to ~679 bp, termed...
To investigate the molecular mechanisms underlying CpG ODN-mediated decrease in TLR9 mRNA, as shown in Fig. 2, the effects of CpG ODN and other compounds on TLR9 gene promoter activity were examined using RPMI 8226 cells transfected with pGL
Naive (Fig. 5). When cells were treated with LPS or K3, luciferase activity levels were significantly decreased, as expected (LPS, 24 ± 1%, Fig. 5a; K3, 46 ± 3%, Fig. 5b). Treatment of cells with INF-γ, CQ, D-type CpG ODN (D19), control ODNs (K3-38p and 1471), or suppressive ODNs (H154 or A151) did not affect luciferase activity levels. TLR9 gene promoter suppression by K3 was reversed by simultaneous treatment with H154, A151, or CQ, whereas the effect of K3 was not reversed by treatment with 1471 (Fig. 5, c–e). CQ did not affect LPS-mediated suppression of promoter activity (Fig. 5). These results suggested that both LPS/TLR4/RP105- and CpG DNA/TLR9-mediated signaling pathways were involved in the suppression of TLR9 gene expression in RPMI 8226 cells.

Identification of transcription factors interacting with essential cis-acting elements

To identify the transcription factors that interact with the cis-acting promoter elements, EMSA targeting R1, R2, R3, or R4 was performed (Fig. 6). When the R1 probe, which spanned the CREB site (−686 to −679 bp), was incubated with nuclear extracts obtained from RPMI 8226 cells, two complexes were detected (Fig. 6A). These complexes were specific for the R1 probe as the band intensity in the nuclear extracts decreased after preincubation with a 10- or 100-fold excess of unlabeled R1 probe, but not with unlabeled mutated R1 probe (mutR1; Fig. 6C). Amounts of both complexes were decreased in nuclear extracts from cells treated with K3, but not with LPS. These complexes were not observed in nuclear extracts from cells preincubated with anti-CREB1, but were present after treatment with anti-ATF2 Ab. This suggested that CREB1 constitutively bound R1, which dissociated after CpG ODN stimulation (Fig. 6, A and B). EMSA also demonstrated two specific complexes for the R2 probe that contained the 5′-PU box site (−252 to −247 bp), after incubation with nuclear extracts from control cells (Fig. 6, D and F). Interestingly, the upper complex was not observed in nuclear extracts from cells treated with K3, but was present after LPS treatment. Preincubation of the nuclear extracts with Ab against various PU box binding proteins, such as Ets2, Elf1, or Elk1, resulted in decreased formation of both complexes, which suggested that Ets2, Elf1, and Elk1 bound to R2. Preincubation of nuclear extracts with anti-Spi1 (PU.1) Ab did not affect complex formation, which suggested that Spi1 (PU.1) was not involved in the complexes (Fig. 6E). A single specific complex was detected using a probe for R3, which contained the 3′-PU box site (−123 to −118 bp) (Fig. 6G). This complex was not affected by preincubation with Ab against PU box binding proteins, such as Ets, Elf1, Elk1, or Spi1 (PU.1) (Fig. 6H). Moreover, the R3 complex was not competed with a 5- or 25-fold excess of mutated R3 probe (mutR3) or R2 probe, which suggested that an unknown protein bound to R3 (Fig. 6I). For the R4 probe, which contained the C/EBP site (−93 to −85 bp), two specific complexes were detected (Fig. 6, J and L). The amount of the upper complex decreased and the lower complex increased in nuclear extracts from cells treated with K3, but not with LPS. Of the anti-C/EBP family Abs tested, preincubation of nuclear extracts with anti-C/EBPα Ab resulted in decreased intensity of both complexes, which suggested that C/EBPα was present in the R4 complexes (Fig. 6K).

Characterization of trans-activity of the hTLR9 gene promoter by known transcription factors

As shown above, several transcription factors appeared to be involved in the regulation of the hTLR9 gene promoter. We next tried to characterize the effect of these transcription factors. An overexpression study was performed using expression vectors for CRE-associating proteins (CREB1 and ATF2), PU box-binding proteins (Ets1, Ets2, Elf1, Elk1, Spi1 (PU.1), SpiB, and SpiC), C/EBPs (C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, and C/EBPε), and proteins up-regulated by CpG DNA or LPS signaling (NF-κB p65, c-Fos, and c-Jun). Cotransfection of cells with pGL Naive plus a vector expressing CREB1, C/EBPα, C/EBPβ, C/EBPγ, or C/EBPε alone resulted in significantly increased luciferase activity compared with cells cotransfected with pGL Naive plus an empty vector (Fig. 7, Ab–Af). In contrast, cotransfection with pGL Naive plus a vector expressing Spi1 (PU.1), c-Jun, or NF-κB p65 alone resulted in decreased luciferase activity (Fig. 7, Aa, Ag, and Ah). The expression levels of these transcription factors were confirmed by Western blot analysis (Fig. 7A). The cooperative effects of combinations of CREB1, Ets2, Elf1, Elk1, and C/EBPα on trans-activation of the TLR9 gene promoter were also examined. Cotransfection with vectors expressing CREB1 and Ets2 increased the luciferase activity compared with cotransfection with vectors expressing CREB1 alone (Fig. 7Ba) or Ets2 alone (Fig. 7Bd), which suggested that CREB1 and Ets2 synergistically trans-activated the promoter.

Similar synergistic enhancements were observed between CREB1 and Elf1 (Fig. 7, Bb and Bc), CREB1 and C/EBPα (Fig. 7, Bc and Bf), Ets2 and Elf1 (Fig. 7, Bg and Bh), Elf1 and C/EBPα (Fig. 7, Bk and Bl), and Elk1 and C/EBPα (Fig. 7, Bm and Bn). Addition of a vector expressing CREB1 to each double combination of transfectant vectors expressing Ets2, Elf1, Elk1, and C/EBPα resulted in increased luciferase activity (Fig. 7, Bo–Bt).
Similar synergistic trans-activation was observed among Ets2, Elf1, and Elk1 (Fig 7, Bu, Bx, and Bw), among Ets2, Elf1, and C/EBPα (Fig 7, By, Bz, and Bc), and among Elf1, Elk1, and C/EBPα (Fig 7, BA–BC). However, addition of Ets2 to a combination of Elk1 and C/EBPα did not change promoter activity. Trans-activation by quadruple combinations of the transcription factors was also examined. Elf1 enhanced the activity induced by Ets2, Elk1, and C/EBPα (Fig 7BHI), whereas CREB1 markedly enhanced the promoter activity induced by any triple combination of Ets2, Elf1, Elk1, and C/EBPα (Fig 7, BA–BC). Finally, the removal of CREB1, Ets2, Elf1, Elk1, or C/EBPα resulted in a significantly decreased promoter activation compared with cells expressing all five factors (Fig 7, BI–BM). These results suggested that the participation of the transcription factors CREB1, Ets2, Elf1, Elk1, and C/EBPα culminated in the maximal transcription of the TLR9 gene.

**Discussion**

This study demonstrated that interactions between CREB1 and the CRE, the Ets2-Elf1-Elk1 complex and the 5'-PU box, and C/EBPα and the C/EBP site critically regulated the hTLR9 gene promoter. Overexpression of known transcription factors suggested that: 1) CREB1 and C/EBPα potently trans-activated the TLR9 gene promoter, 2) although Ets2, Elf1, and Elk1 did not trans-activate the promoter themselves, they enhanced the trans-activity of factors acting on other elements, 3) Spi1 (PU.1) acted, directly or indirectly, as a suppressor on the TLR9 gene promoter, 4) factors up-regulated by CpG DNA/TLR9 or LPS/TLR4/RP105 signaling (NF-κB and c-Jun, a component of AP-1) also acted as suppressors, and 5) other members of the C/EBP family, including C/EBPβ, C/EBPδ, and C/EBPε, may also be involved in the up-regulation of TLR9 gene expression in other cell types or via other stimuli.
CREB1 is expressed in a wide variety of cell types. It has been established that cAMP induces phosphorylation of CREB1, which then activates cAMP-responsive genes, leading to increased cell proliferation, differentiation, or modulation of various cell functions (24). In B cells, CREB1 phosphorylation is enhanced by cell surface Ig cross-linking (25).

The Ets family of transcription factors is a diverse group of ~30 proteins that share a conserved DNA binding domain. Most Ets family members bind to PU box/Ets sites within gene promoters. Ets2 is expressed in a variety of tissues, and transgenic animal studies have demonstrated that Ets2 plays a critical role in thymocyte and macrophage development (26). Elf1 is a lymphoid-specific Ets transcription factor that regulates the germline Ig α gene promoter in B cells and IL-3 and IL-2Rα gene expression in T cells (27–29). Elf1 has been shown to cooperate with C/EBPα to upregulate promoter activity of the Igα FcR (CD89) gene in the monocytic cell line U937 (30). Another family member, Elk1, is a direct target of MAPKs, such as ERK1/2. Once phosphorylated, it translocates to the nucleus and serves as a transcription factor (31). Physical interaction between Elk1 and C/EBPαβ confers insulin sensitivity in Chinese hamster ovary cells and synergistic transactivation of the c-fos gene (32–34). Constitutive and sustained Elk1 phosphorylation after cell surface Ig cross-linking has been reported in mature B cells (25).

C/EBPs belong to the basic region/leucine zipper class of transcription factor and are involved in differentiation in a broad range of tissues. C/EBPα expression levels tend to be prominent in immature myeloid cells and can vary during differentiation. C/EBPα is essential for granulocyte development and blocks Spi1 (PU.1)-induced dendritic cell development from CD34+ human cord blood cells (35). C/EBPα has been shown to cooperate with other transcription factors to enhance or suppress promoter trans-activity. The leucine zipper in the DNA binding domain of C/EBPα physically binds to Ets transcription factors, such as Spi1 (PU.1), Fli1, Ets1, and Elk1 (36). Due to the close proximity of the C/EBP site to the TATA box within the TLR9 gene promoter (Fig. 4), it is possible that C/EBPα may interact with the basal transcription-initiation complex. Indeed, CBP/p300, which forms the basal transcription apparatus by interacting with the TATA box-binding protein TFIIB and RNA polymerase II, forms a binding domain for C/EBPαβ, CREB, and Ets family transcription factors (24). Thus, CBP/p300 may play a central role in the transcriptional regulation of the TLR9 gene by controlling the interaction between various trans-acting factors. Taken together, our findings suggest that CREB1, Ets2, Elf1, Elk1, and C/EBPαβ might physically and functionally interact with each other, leading to maximal transcription of the TLR9 gene.

Overexpression of Spi1 (PU.1) resulted in suppression of the TLR9 gene promoter (Fig. 7A). Although functional redundancy has been demonstrated between Spi1 (PU.1) and SpiB, other members of the Ets family, such as Ets1 or Elf1, are not functionally similar to Spi1 (PU.1) (37). Thus, it is possible that overexpressed...
PU.1 might compete with endogenous Ets2, Elf1, and Elk1 for PU box binding rather than serving as a trans-activator for the TLR9 gene promoter, or alternatively, PU.1 might directly interact with endogenous transcription factors, blocking their trans-activity. In a case of the I-A β gene expression, Spi1 (PU.1) competes with other transcription factors for DNA binding, which results in transcriptional suppression (38). It has also been demonstrated that the development of dendritic cells from cord blood stem cells transduced with Spi1 (PU.1) was blocked by C/EBPα through a physical interaction (35). Thus, it is likely that Spi1 (PU.1) suppressed C/EBPα-mediated activation of the TLR9 gene promoter via a protein-protein interaction.

Tissue- and developmental stage-specific expression of a given gene is not achieved through the action of a single transcription factor. Rather, unique combinations of cell type-specific and more generally expressed nuclear factors account for the enormous specificity and diversity in gene expression profiles (24). For instance, expression of the hTLR2 gene in monocytic cells is regulated by Spi1, Sp3, and Spi1 (PU.1), whereas hTLR4 gene expression is regulated by IFN consensus sequence binding protein and Spi1 (PU.1) (39, 40). Interestingly, hematopoietic progenitor cells from Spi1 (PU.1) gene-knockout mice are unable to generate myeloid dendritic cells (MDCs) in vitro, which express high levels of TLR2 and TLR4, but not TLR9 (41). With regard to its role in hTLR9 gene expression, Spi1 (PU.1) might play a key role in regulating the cell type-specific profile of TLR family gene expression.

Species-specific regulation of TLR family gene expression has recently been demonstrated. Murine TLR2 gene expression is up-regulated by LPS, mycobacterial products, and NF-κB activation, whereas hTLR2 gene expression is not induced by these stimuli or by NF-κB activation. Indeed, the mTLR2 gene promoter contains an NF-κB motif, which functions as a positive cis-acting element (42). Basal and IFN-β-induced activation of the TLR3 gene promoter from mice and humans involves similar IFN regulatory factor (IRF) elements that constitutively bind IRF-2 and recruit IRF-1 after stimulation. However, in mouse macrophages, LPS up-regulates TLR3 gene expression through IFN-β in an autocrine manner, whereas in human MDCs, LPS blocks the IFN-β-induced up-regulation of TLR3 (43). Comparison of the 5’-flanking regions between mTLR9 and hTLR9 genes showed 56% homology and both of them contained the CRE, the PU box, and the C/EBP site (Fig. 4).

PDCs show the highest level of TLR9 gene expression and can be generated in vitro from CD34+ hematopoietic progenitors. Exogenous overexpression of Id2 or Id3, transcription inhibitors with basic helix-loop-helix structures, inhibits the development of progenitors into PDCs, but not into MDCs (44, 45). The Ets family transcription factor SpiB is abundantly expressed in hematopoietic progenitors and PDCs, but not in other myeloid/lymphoid cells, including MDCs. Moreover, it has been suggested that SpiB is involved in the control of PDC development by limiting the capacity of progenitor cells to develop into other lymphoid lineages (46). Although SpiB overexpression did not alter TLR9 gene promoter activity in RPMI 8226 cells (Fig. 7A), other mechanisms involving SpiB, Id2, or Id3 may modulate TLR9 gene expression in PDCs.

A recent study by Hornung et al. (18) demonstrates that the B type (also known as K type) CpG ODN 2006 suppressed TLR9 gene expression in both primary B cells and PDCs. However, this study did not further clarify the mechanism underlying the TLR9 gene suppression by CpG DNA. In the present report, we demonstrate that treatment of RPMI 8226 cells with K type CpG ODN (K3) suppressed the DNA binding activity of CREB1, Ets2, Elf1, Elk1, and C/EBPα to their respective cognate cis-acting elements, which then reduced promoter activity and resultant TLR9 gene mRNA levels. The CpG ODN-mediated suppression of TLR9 gene promoter was further confirmed by the use of CpG-specific inhibitors, such as suppressive ODNs and CQ (22, 47). This effect was also observed after overexpression of the transcription factors c-Jun or NF-κB p65, which mediate CpG DNA-induced cytokine/chemokine gene expression (20, 47). This indicated that these factors and/or gene products up-regulated by CpG DNA were, directly or indirectly, involved in the mechanism underlying the TLR9 gene suppression. However, this finding contradicts studies independently reported by Bourke et al. (48) and Bernasconi et al. (49), in which activation by surface Ig cross-linking, Staphylococcus aureus Cowan I bacteria, or B-type CpG ODN 2006 increased TLR9 expression in primary human B cells. However, both groups used tonsillar resting or naive B cells to study TLR9 gene expression. These immature B cells express lower levels of TLR9 than do activated mature B cells, such as germinal center or memory B cells. Also, the responsiveness to B cell Ag-receptor complex or polyclonal B cell activators, such as LPS or CpG DNA, is dependent on differentiation stage and can vary from donor to donor. Indeed, we tested several human B cell lines at different stages of differentiation and found that only RPMI 8226 cells expressed the TLR9 gene after treatment with CpG ODN or LPS (data not shown). More precise studies will be needed to clarify the relationship between modulation of TLR9 gene expression by CpG DNA and B cell differentiation stage.

In this study, we identify four cis-acting elements, CRE, 5’-PU box, 3’-PU box, and C/EBP site, that synergistically regulate the hTLR9 gene promoter. CREB1, Ets2, Elf1, Elk1, and C/EBPα trans-activate the promoter by interacting with these cis-acting elements. Synergism between the cis- and trans-factors appear to account for the maximal hTLR9 gene transcription. Our findings will extend our understanding of cell type-specific TLR9 gene expression and will assist in the development of therapeutic strategies able to modulate responsiveness to CpG ODN for clinical purposes.

References


