Transcription Factor PU.1 Controls Transcription Start Site Positioning and Alternative TLR4 Promoter Usage*§

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Human and mouse show markedly different sensitivities toward bacterial endotoxins, and recent evidence suggests that a species-specific regulation of the lipopolysaccharide receptor Toll-like receptor 4 (Tlr4) may contribute to this phenomenon. To gain further insight into mechanisms of Tlr4 regulation, we conducted a detailed in vivo and in vitro study of the murine Tlr4 gene, including analysis of transcription start site location, transcription factor occupancy, and activities of its proximal regulatory sequences. Our analyses identified a PU.1-dependent myeloid promoter, which is conserved between humans and mouse. We also identified an additional, distal promoter, located ~200 bp upstream of the myeloid-specific promoter, which is a functional target of E-box binding factors. In contrast to humans, where non-myeloid cells utilize both promoters, the distal Tlr4 promoter initiates all Tlr4 transcripts in murine non-myeloid cells, indicating that species-specific differences in TLR4 mRNA regulation may primarily exist in non-myeloid cell types. Interestingly, PU.1 null murine myeloid progenitor cells predominantly use the distal promoter, and the conditional induction of PU.1 expression in these cells leads to the rapid switch of transcription initiation to the proximal myeloid promoter. This indicates a direct role for PU.1 in determining the transcriptional start site and in recruiting the basal transcription machinery to myeloid promoters.

Instead, they contain (often multiple) DNA binding motifs for the myeloid- and B-cell-specific transcription factor PU.1, which plays an important role in regulating macrophage development and macrophage-specific gene expression (4–6). Earlier in vitro protein-protein interaction studies indicated that PU.1 may physically associate with the transcription factor TFIID (7), a property that may be important for the recruitment of the basal transcription machinery in the absence of a conventional TATA box or INR element. A link between PU.1 and transcription initiation is also suggested by a number of studies investigating macrophage-specific promoters. It has been shown, for example, that an artificial assembly of ETS factor binding sites is sufficient to drive the macrophage-specific expression of a reporter gene (8). PU.1 is also necessary and sufficient to activate transcription from the macrophage-specific c-fms promoter (9). However, it is unclear whether the presence or absence of PU.1 determines the transcriptional start site to which the basal transcription machinery is recruited.

The Toll-like receptor 4 (TLR4) genes in human and mouse represent prime examples for macrophage-specific gene regulation; proximal promoters lack TATA boxes and contain multiple binding sites for PU.1. With few exceptions, TLR4 is transcribed in PU.1-expressing cells, including mononuclear phagocytes and B-cells. The protein encoded by TLR4 is crucial for endotoxin signaling and is a limiting factor for the innate response to bacterial lipopolysaccharide (LPS) (11). Recognition and response to LPS requires tight regulation to achieve sufficient immune activation and to prevent septic shock. Interindividual or interspecies variations in the expression and regulation of the integral signaling component TLR4 may provide an explanation for observed differences in LPS sensitivity, e.g. between mouse and human (12). In support for this hypothesis, a number of recent studies used TLR4 transgenic mice to demonstrate that Tlr4 expression levels (depending on the transgene dosage) determine the extent of innate responses to LPS (13–15).

Recent evidence from promoter studies by us and another group (10, 16, 17) suggested a strikingly different regulation of TSS, transcription start sites; IEC, intestinal epithelial cells; BMM, bone marrow-derived macrophages; CHOn, 4-hydroxytamoxifen; RLM, RNA ligase mediated; RACE, rapid amplification of CDNA ends; pol, polymerase; DMS, dimethyl sulfate; EMSA, electrophoretic mobility shift assay; IC50, half-maximal inhibitory concentration; mWT, murine wild-type construct; dMWT, distal mouse Tlr4 promoter.

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¶ The abbreviations used are: INR, initiator; TF, transcription factor; TLR, Toll-like receptor; LPS, lipopolysaccharide; USF, upstream stimulatory factor.
TLR4 genes in mouse and human that may also influence the innate response to LPS in both species. Reporter gene analyses suggested that different cis-regulatory sequences may control the transcription of human and murine TLR4 genes, although promoter sequences are largely conserved in both species (10).

To gain a better understanding of the different regulatory mechanisms controlling the macrophage-specific expression of TLR4 in both species, we performed a detailed analysis of the mouse Tlr4 promoter in comparison with its human orthologue. Using reporter assays, we demonstrate repressory effects of mouse-specific out-of-frame ATGs that likely influence TLR4 expression on translation levels. We also show, for the first time, that Tlr4 is transcribed from two alternative promoters. Using in vivo footprinting and chromatin immunoprecipitation techniques, we demonstrate that the murine myeloid-specific proximal promoter, like its human counterpart, primarily binds the myeloid- and B-cell-specific transcription factor PU.1. We also identify an additional, alternative distal promoter, which contains an integral E-box that is bound by E-box binding factors such as USF and/or Mitf/TFE family transcription factors. Murine, non-myeloid cell types such as epithelial cells or fibroblasts utilize the distal, but not the proximal, myeloid promoter for Tlr4 transcription, whereas human epithelial cells use both promoters. We also show that the conditional activation of PU.1 in a hematopoietic progenitor cell line (18) results in the rapid alteration of transcription start sites (TSSs) and enhanced recruitment of polymerase II to the proximal Tlr4 promoter, providing direct evidence that PU.1 is instrumental in determining promoter usage and in directing the location of the transcription initiation complex.

EXPERIMENTAL PROCEDURES

**Chemicals**—All chemical reagents used were purchased from Sigma-Aldrich (Berlin, Germany) unless otherwise noted. Protease inhibitors were from Roche Applied Science. Oligonucleotides were synthesized by Operon (Cologne, Germany) or Metabion (Martinsried, Germany). Antiseria for supershift analyses were purchased from Santa Cruz Biotechnology.

**Cells**—Monocytes were isolated and cultured to generate macrophages as described earlier (19). Human intestinal epithelial cells (IEC) were prepared from colonic tissue obtained from patients undergoing surgery for colorectal carcinoma. The mucosa was incubated in 1 mM dithiothreitol to remove the mucus, and cells were collected after shaking in 2 mM EDTA (20). Murine bone marrow-derived macrophages (BMM) and intestinal epithelial cells (m-IC12, IEC) from different mouse strains (C57Bl/6, BALB/c) were cultured as described previously (8, 21, 22). The human monocytic cell line THP-1 and the human colon adenocarcinoma cell line HT29, the murine macrophage cell line RAW264.7 were maintained in RPMI 1640 medium plus 10% fetal calf serum and supplements. The human colon adenocarcinoma cell line HCT29, the murine fibroblast cell line NIH 3T3, and colon carcinoma cell line CT26 were cultured in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum and supplements. PUER cells (18) were cultivated in Iscove's modified Dulbecco's medium (Invitrogen), supplemented with penicillin/streptomycin (10,000 units/ml), glutamine (200 mM), β-mercaptoethanol (50 μM), mouse interleukin-3 (5 ng/ml; BIOSOURCE), puromycin (1 μg/ml), and 10% fetal calf serum. To activate the PU.1-ER fusion protein, PUER cells were centrifuged and resuspended in medium containing 0.1 μM tamoxifen (OHT).

**RNA Preparation, Real-time PCR**—Total RNA was isolated with the RNeasy mini kit (Qiagen) or with phenol-chloroform extraction as described by Chomczynski and Sacchi (23). 1 μg of RNA was reverse-transcribed using SuperScript II Moloney murine leukemia virus reverse transcriptase (Invitrogen or Promega). Real-time PCR was performed on a LightCycler (Roche Applied Science) or a realplex Mastercycler EP (Eppendorf) using the Quantitect kit (Qiagen) according to the manufacturer’s instructions. Primers are listed in supplemental Table 1. Cycling parameters were: denaturation 95 °C, 15 min, amplification 95 °C, 15 s, 56 °C, 20 s, 72 °C, 25 s, for 45 cycles. Melting curves were analyzed to control for specificity of the PCR reactions. Data were normalized for expression of the housekeeping gene HPRT. The relative units were calculated from a standard curve plotting three different concentrations of log dilutions against the PCR cycle number at which the measured fluorescence intensity reaches a fixed value. The amplification efficiency E was calculated from the slope of the standard curve by the formula: E = 10^(-1/slope). For each sample, data of at least three independent analyses were averaged.

**5’-RLM-RACE PCR**—Transcription start sites were determined by using 1 μg of total RNA for cDNA synthesis with the FirstChoice™ RLM-RACE Kit (Ambion). Gene-specific primers were used to amplify endogenous full-length 5′-cDNA fragments of human or murine TLR4, as well as murine Lpl, Chi3l3, and Cd14. Transcription start sites of luciferase reporter genes were detected using luciferase gene-specific primers. PCR products were cloned into pCR2.1-TOPO vector (TOPO cloning kit, Invitrogen), and inserts from at least 10 individual plasmid-containing bacterial colonies derived from each RLM-RACE PCR reaction were reamplified by PCR and directly sequenced (performed by GENEART, Regensburg, Germany). Primer sequences are listed in supplemental Table 1.

**Plasmid Construction and Purification**—Construction of the human TLR4 reporter was previously described (10). Initially, an 1800-bp fragment of the murine promoter was amplified from a murine bacterial artificial chromosome clone using the Expand high fidelity PCR system (Roche Applied Science) and subcloned into the pGL3 vector. The Tlr4 promoter construct used in this study was created by SmaI/EcoRV digest and subsequent religation. Mutations and chimeras of the proximal Tlr4 construct were generated by overlap PCR using specific primers (sequences are either described (10) or listed in supplemental Table 1) together with the vector-specific primer GL2 and RV3 (Invitrogen). The obtained PCR fragments were subcloned with MluI and NcoI into pGL3-B or pGL4-B (Promega). DNA sequence analysis was performed by Entelechon (Regensburg, Germany). For transfections, plasmids were isolated and purified using the Endofree plasmid kit from Qiagen.

**Transient DNA Transfections**—NIH 3T3 and RAW264.7 cells were transfected in duplicates using Lipofectamine (Invitrogen) according to the manufacturer’s protocol. 0.2 × 10⁶ cells were seeded into each well of a 12-well plate. For NIH 3T3 transfection, 0.2 μg of the firefly reporter plasmid and 10
ng of the Renilla plasmid were used in each transfection, and for RAW264.7, 2 μg and 50 ng were used in each transfection. In cotransfection analysis, the amount of DNA was split equally between reporter and expression plasmid. After 24 h, cell lysates were assayed for firefly and Renilla luciferase activity using the Dual-Luciferase reporter assay system (Promega) on a Sirius luminometer (Berthold). Firefly luciferase activity of individual transfections was normalized against Renilla luciferase activity. Expression plasmids for dominant negative A-Mitf and A-USF (24) as well as wild-type TFEB, TFE3 (25), USF1, and USF2a (26) were described previously.

Nuclear Extracts and Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared as described (10). Double-stranded oligonucleotides corresponding to the PU.1 and E-box elements were labeled with α-[32P]dATP or α-[32P]dGTP using Klenow DNA polymerase. Oligonucleotide sequences are aligned in supplemental Figs. 3 and 5B. The binding reaction contained 2.5 μg of nuclear extract protein, 0.5 μg of poly dI/dC, 20 mM HEPES, pH 7.9, 20 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, pH 8.0, 5% glycerol, and 20 nmol of probe DNA in a final volume of 10 μl. Antisera used in supershift analyses were added after 15 min, and samples were loaded onto polyacrylamide gels after incubating at room temperature for a total of 30 min. Buffers and running conditions used have been described before (10). Gels were fixed in 5% acetic acid, dried, and autoradiographed.

Chromatin Immunoprecipitation—ChIP analysis was performed with modifications of previous described methods (27). Cells were fixed with 1/10th volume of fixation buffer (500 mM HEPES/KOH, pH 7.9, 0.1 M NaCl, 1 mM EDTA, pH 8.0, 5% formaldehyde) for 10 min at room temperature and stopped by adding glycine (0.125 M final). After two washings in phosphate-buffered saline (with 1 mM phenylmethylsulfonyl fluoride), 10 × 106 cells were resuspended in 250 μl of L1A (10 mM HEPES/KOH, pH 7.9, 85 mM KCl, 1 mM EDTA, pH 8.0) and lysed in 250 μl of L1B (L1A + 1% Nonidet P-40) for 10 min on ice. After centrifugation, 400 μl of nuclear lysate buffer was added and sonicated two times for 10 s on setting 2 (Branson Sonifier 250, Danbury, CT) to obtain around 500-kb chromatin fragments. The lysate was centrifuged, 4 μl of the supernatant was kept as input, and the rest was diluted 2.5-fold with dilution buffer. All buffers contain 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml peptatin, 1 μg/ml aprotonin. For each sample, 200 μl was precleared for 2 h at 4 °C with 50 μl of CL4B, preincubated with 0.05% bovine serum albumin and 5 μg of sheared salmon sperm DNA, and immunoprecipitated with 2.5 μg of each antibody overnight at 4 °C: anti-PU.1, anti-rabbit-IgG, and anti-RNA-pol II (Abcam). Recovering of complexes was done with 30 μl of protein A-Sepharose and 2 μg of sheared salmon sperm DNA for 2 h at 4 °C. The beads were washed in centrifugal filter devices (Millipore) with 400 μl of washing buffer I, II, and III, 2 × Tris/EDTA, and complexes were eluted with 2 × of 100 μl of elution buffer. DNA was recovered with a QiAquick PCR purification kit (Qiagen) in 100 μl of elution buffer (10 mM Tris-HCl, pH 8.5). Quantification was performed with a SYBR green master mix (Roche Applied Science) or realplex Mastercycler EP (Eppendorf) with a primer pair amplifying the −250 to −90 promoter fragment. A Tlr4 upstream and an internal Gapdh gene fragment, which did not show specific transcription factor binding, were used to control for variability between individual precipitations, which was usually negligible (for primer sequences, see supplemental Table 1).

In Vivo Genomic Footprinting with DMS—In vivo DMS footprinting was performed with 1.5 μg of purified genomic DNA from DMS-treated cells as previously published (28). For a control, DMS treatment with following papiridine cleavage was also conducted with naked DNA. Primer pairs for ligation-mediated PCR and second amplification PCR of mouse and human TLR4 promoters are listed in supplemental Table 1. Differences in DMS accessibility were visualized on a denaturing polyacrylamide gel after a labeling reaction with a Cy5 end-labeled-linker primer. Imaging was done directly on a Typhoon scanner (GE Healthcare).

RESULTS

TLR4 Transcription Start Sites in Myeloid and Non-myeloid Cell Types—In contrast to a number of other TLR family members, mammalian TLR4 genes share evolutionary conserved promoter sequences (12). Alignment of TLR4 upstream sequences highlights the presence of several conserved sequence blocks, which include consensus binding elements for the myeloid transcription factor PU.1 (supplemental Fig. 1). However, despite the presence of highly conserved promoter sequences, reporter gene studies by us and others (10, 17) indi-
cated a different promoter structure of human and mouse TLR4 genes, which may explain species-specific variations in LPS responsiveness. The structural differences included the species-specific location of relevant PU.1 sites that could result in differential TLR4 expression. To start characterizing the different nature of human and mouse promoters, we determined the TSS of endogenous Tlr4 genes in human and mouse myeloid and non-myeloid cell types. The results of representative RLM-RACE PCRs are shown in Fig. 1. In case of the human TLR4 gene, endogenous transcripts of myeloid (monocytes and THP-1 cells) and non-myeloid cells (intestinal epithelial cell line HT29 and primary human IEC) mainly initiated at the proximal initiator sequence downstream of a putative NF-Y box, and to some extent, also at a site downstream of the conserved PU.1/ICSBP site. Primary mouse macrophages mainly utilized a TSS downstream of the PU.1/interferon regulatory factor site that was also detected in humans, and to a lesser extent, a TSS corresponding to the major human start site. RAW264.7 macrophages also used a distal TSS that represented the sole start site for all tested non-myeloid murine cell types, including intestinal epithelial cells (CT26, m-ICcl2 and primary murine IEC) and NIH 3T3 fibroblasts. (The exact positions of TSS in sequenced RLM-RACE PCR products are presented in supplemental Fig. 1.)

Transcription Factor Occupancy at the Murine TLR4 Promoter in Bone Marrow-derived Macrophages

To investigate which cis-elements and which transcription factors might contribute to Tlr4 promoter activity in murine macrophages, we investigated transcription factor binding to the proximal Tlr4 promoter by DMS in vivo footprinting. In bone marrow-derived macrophages, hyper- or hyposensitivity toward DMS induced methylation was observed at several putative PU.1 elements (Fig. 2A). We reproducibly detected footprints at the combined PU.1_1/ICSBP, PU.1_2, PU.1_3, PU.1_5, and PU.1_6 sites and next to the PU.1_7 site. No footprint was observed at the PU.1_4 site (data not shown). All corresponding PU.1 sites were occupied in human monocytes and macrophages (see supplemental Fig. 2), indicating that despite their different importance in reporter assays, similar sites may contribute to myeloid promoter activ-
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ity in both species. In addition to the PU.1 sites, macrophages of both species also demonstrated DMS hyperreactivity in the upstream region including the conserved AP-1/E-box element (Fig. 2A and supplemental Fig. 2).

To investigate whether the occupied PU.1 consensus sequences were binding PU.1 or another Ets family member, EMSA studies were performed using radiolabeled probes for every putative PU.1 binding site and nuclear extracts from bone marrow-derived macrophages. PU.1-containing complexes were detected for PU.1_1, -2, -3, -5, and less intense binding was detected for PU.1_4 and -8 (shown in Fig. 2B and supplemental Fig. 3). The absence of significant PU.1 binding at PU.1_6 in EMSA indicates that the corresponding footprints might derive from a nuclear factor other than PU.1, which is in line with the presence of footprints at this site in PU.1-negative non-myeloid cells (see below).

To analyze the functional relevance of the different putative PU.1 binding sites, we performed transient transfections in RAW264.7 macrophages using comparable mouse and human promoter constructs with introduced point mutations in all putative PU.1 binding sites. Luciferase reporter activities of the mouse Tlr4 promoter were ~5-fold lower as compared with the human promoter. In human constructs, two proximal PU.1 sites (PU.1_1 and PU.1_2) were essential for reporter activity, which is in line with our previous findings in human myeloid THP-1 cells (10). However, in the murine Tlr4 reporters, only one distal PU.1 binding site (PU.1_5) was necessary for full reporter activity, which was previously also documented by Roger et al. (17) (supplemental Fig. 4, A and B). Determination of luciferase TSS revealed that luciferase transcripts derived from the mouse Tlr4 reporter gene initiated transcription primarily from the distal (non-macrophage) TSS in RAW264.7 macrophages, whereas human TLR4 reporter genes utilized the correct endogenous TSS of primary macrophages (supplemental Fig. 4C). Reasons for the apparent discrepancy between TSSs in endogenous and reporter genes in the case of murine Tlr4 (but not human TLR4) are currently unknown but may include vector-related issues or a different chromatin architecture in transiently transfected templates as compared with that of the endogenous gene as observed in Smith et al. (29). Our results strongly suggest that the reporter gene analyses performed in this and a number of previous studies (16, 17, 30) primarily inform about regulatory properties of the distal non-myeloid promoter and question the relevance of transient reporter studies for the proximal myeloid promoter that is predominantly used in murine macrophages.

To further prove the specific binding of PU.1 in vivo, we performed ChIP assays for PU.1 in bone marrow-derived macrophages. We also performed a ChIP assay for RNA-polymerase II to confirm that the presence of PU.1 parallels the establishment of an active promoter. The results are shown in Fig. 2C. PU.1 was strongly enriched at the promoter, which is consistent with the large number of occupied PU.1 binding sites.

In summary, the above data suggest that PU.1 plays a major role in TLR4 regulation in both human and murine macrophages, most likely through a set of conserved binding sites.

Characterization of the Distal Tlr4 Promoter—The structure of the newly defined distal promoter of the murine Tlr4 gene, which was predominantly utilized in non-myeloid cells, was further analyzed in vivo and in vitro. To investigate which cis-elements might contribute to Tlr4 promoter activity in murine non-myeloid cells, we analyzed Tlr4-expressing non-myeloid NIH 3T3 and CT26 cells by DMS in vivo footprinting (Fig. 3). In contrast to DMS footprints in RAW264.7 macrophages (which resembled BMM footprints shown in Fig. 2A), alterations in DMS reactivity in both murine non-myeloid cell types were restricted to the region upstream of the distal TSS containing the AP-1/E-box motif. Next, we generated reporter constructs harboring distal promoter sequences and mutated putative transcription factor binding sites. Results of transient transfection assays with RAW264.7 and NIH 3T3 cells are shown in Fig. 4A. In both cell types, we observed a strong decrease of luciferase activity in the constructs that contain mutations of the highly conserved AP-1/E-box motif (dmA/E1), indicating an important role of this element in maintaining high level promoter activity. Mutation of the dmPU.1_5 binding site resulted in a significant (50%) reduction of reporter activity in RAW264.7 macrophages, whereas having only a marginal effect in NIH 3T3 fibroblasts.

To detect which factors might bind the footprinted region of the AP-1/E-box element, we performed EMSAs using nuclear extracts (NE) from NIH 3T3 and RAW264.7 (Fig. 4B). Previous studies claimed that this site was bound by AP-1 family proteins. However, consensus motifs for AP-1 (or the related cAMP-response element-binding protein) did not act as competitors in EMSA (Fig. 4B, lanes S and 13). Instead, consensus
motifs for E-box binding factors Mitf (M-box), TFE3, and USF (Fig. 4B, lanes 14–16) efficiently competed for protein binding to the AP-1/E-box element, and a major fraction of the detected protein-DNA complexes in NIH 3T3 and RAW264.7 nuclear extracts was supershifted in the presence of antibodies against E-box binding factors USF1 or USF2 (Fig. 4B, lanes 18 and 19).

Although most complexes were supershifted by USF factor antibodies in RAW264.7 cells (shown in supplemental Fig. 5), two yet unidentified complexes (C and D) were specifically detected in NIH 3T3 fibroblast. Cold competition studies with mutated AP-1/E-boxes (mA/E) or consensus sequences for the transcription factors AP-1, cAMP-response element-binding protein, Mitf, USF, and TFE3 showed that USF-containing complexes required an intact E-box core (mutations at A/E1 and A/E2), and to some extent, an adjacent GT-dinucleotide (mA/E3). Competition for complex C required intact sequences A/E1 and A/E3. Complex D appears to require A/E3 and A/E6; however, the requirement for A/E3 was diminished when A/E1 was also mutated.

To identify which E-box-binding protein family was able to regulate the distal TLR4 promoter, we performed co-transfection experiments with expression plasmids for USF1, USF2, and...
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A

B

C

FIGURE 5. Role of PU.1 in determining TSSs in PUER cells. A, real-time PCR for Tlr4 expression in PUER cells at the indicated time points after OHT treatment. Results are normalized for Hprt expression and relative to unstimulated cells (0 h). Values are means ± S.D. of two independent experiments, analyzed in duplicates. B, analysis of Tlr4 TSSs during a time course of OHT induction. The arrows indicate the major start sites in the absence (TSS distal) or presence of PU.1 (TSS prox.). C, ChIP analysis for Pol II, PU.1, binding at the Tlr4 promoter (−250 to −90) during OHT treatment in PUER cells. Values are means ± S.D. of two independent experiments, analyzed in duplicates.

FIGURE 6. Species-specific role of out-of-frame ATGs. The consequences of mutation (Ch1m, ATGm) or insertion (Ch2m) of two mouse-specific S'-ATGs were analyzed using transient transfection in RAW264.7 macrophages. Constructs are shown schematically. Mouse sequences are represented by black boxes, and human sequences are represented by white boxes. Positions of ATG triplets are indicated by an M. Values are relative to the human wild-type (hWT) construct (100%, not shown) and are means ± S.D. of (at least) three independent experiments.

the two microphthalmia TFE family members TFE3 and TFE3 that were previously shown to be expressed in NIH 3T3 cells (31). These demonstrated that TFEB and TFE3 but not USF1 or USF2 trans-activated the wild-type Tlr4 luciferase reporter gene in NIH 3T3 and RAW264.7 cells (Fig. 4C). This was independently confirmed by the expression of dominant-negative factors that specifically block the binding of either USF or microphthalmia TFE family factors. Co-transfections in both cell lines resulted in a dose-dependent reduction of Tlr4 reporter gene activity only in the case of A-Mitf, the dominant negative inhibitor of microphthalmia TFE family transcription factors, but not with A-USF (Fig. 4D). Chip-grade antibodies against TFEB and TFE3 were unavailable. Significant in vivo binding of USF1 and USF2 was detected in BMM, and to a lesser extent, in RAW264.7 macrophages, but not in NIH 3T3 fibroblasts (data not shown).

These experiments established an important role for the conserved E-box element in the regulation of the distal promoter. Despite their prominent binding in EMSA studies, USF factors appear to play a minor role in Tlr4 gene regulation. Co-transfection studies in Fig. 4, C and D, indicate a role for microphthalmia TFE members TFE3 and TFEB. However, additional studies will be needed to clarify the exact role of individual E-box binding factor families and the identity of complexes C and D that may play an important role for Tlr4 expression in non-myeloid cells.

The Presence or Absence of PU.1 Determines the Transcription Start Site—One difference between myeloid and non-myeloid cells is the presence of PU.1, raising the possibility that PU.1 could be directly involved in determining the TSS in myeloid cells. To test this hypothesis, we took advantage of the hematopoietic progenitor cell line PUER that was originally generated from an established interleukin-3-dependent PU.1−/− progenitor line by retroviral expression of an inducible PU.1-ER fusion protein (18). In this cell line, the addition of tamoxifen (OHT) activates DNA binding of PU.1 to target promoters. We first analyzed Tlr4 mRNA expression during a 48-h time course of PU.1 induction in these cells. As shown in Fig. 5A, Tlr4 expression gradually increased over time, and a 2-fold induction of mRNA expression was already detected after 30 min of OHT treatment. We next analyzed the position of TSS during the time course. As shown in Fig. 5B, non-induced PUER cells utilized the distal non-myeloid promoter. Upon induction with OHT, we observed a continuous shift from the distal non-myeloid toward the proximal myeloid TSSs within 1 day. Again, changes were already detected during the first 30 min, suggesting that PU.1 was responsible for the promoter switch. Other PU.1 target genes including Chi3l3, Cd14, and Lpl did not show changes in the position of TSSs (see supplemental Fig. 6). We analyzed the recruitment of PU.1 to the proximal promoter in vivo by ChIP assays and also examined the recruitment of RNA-Pol II after stimulation (Fig. 5C). As compared with the IgG ChIP we saw a strong recruitment of PU.1 to the promoter already after 15 min. Simultaneous binding of RNA-Pol II increased after 15 min, indicating more active transcription.

In concert with the earlier observation that PU.1 can interact with TFIIID in glutathione S-transferase pulldown assays, our observations suggest that PU.1 may determine the TSS by direct recruitment of the transcription initiation complex to a myeloid promoter. This property of PU.1 may, however, be
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**FIGURE 7. Schematic illustration of TLR4 promoter structures in mouse and human.** Transcription factor occupancy and recruitment of the basal transcription machinery are indicated for TLR4 promoters in bone marrow-derived macrophages (myeloid) and non-myeloid fibroblasts or intestinal epithelial cells. Upstream PU.1 (5) and AP-1/E-box elements are highly conserved and likely bound by similar factors (PU.1 and basic helix-loop-helix factors such as USF or Mitf/TFE, respectively) in mouse and human.

Dependent on the promoter context including the presence or absence of strong initiator elements or TATA boxes.

**Impact of Species-specific Out-of-Frame ATGs—**During the course of our study, we performed numerous reporter assays where we observed that the proximal 5′-untranslated region non-coding and coding mouse sequences showed strong repressor effects in chimerical constructs of human and mouse sequences (Fig. 6 and data not shown). In this region, we noted the presence of two out-of-frame-ATGs, which were absent from the human sequence. Since out-of-frame ATGs are known suppressors of post-transcriptional gene expression (32), we investigated the function of the murine-specific out-of-frame-ATGs in reporter assays. As shown in Fig. 6, insertion of these ATGs into the human sequence context (Ch2 → Ch2m) reduced reporter activity, whereas removing them from the murine wild-type construct (mWt → ATGm) or the chimerical human/mouse construct (Ch1 → Ch1m) markedly induced reporter gene activity. The above results indicate that out-of-frame-ATGs may influence translation efficiencies of Tlr4 transcripts in a species-specific manner and at least partially contribute to the observed difference in basal reporter gene activities measured for human and murine Tlr4 constructs.

**DISCUSSION**

TLR4 is an integral component of our immune system and is required for the innate recognition of bacterial LPS. Our previous study indicated that the cell type-specific regulation of the human TLR4 gene is controlled by a typical myeloid promoter. PU.1 was identified as a major regulator of TLR4, which is consistent with its pivotal role in myeloid gene regulation. Reporter gene studies by us and other groups indicated a different structure of the Tlr4 promoter in mice (10, 16, 17). In consideration of the high degree of sequence conservation between mouse and human 5′-sequences, this observation was surprising but could explain the different TLR4 expression pattern observed in both species. Human TLR4 expression is mainly detected in the spleen, peripheral blood monocytes, and neutrophils, whereas in mice, Tlr4 mRNA is found more broadly (33). The present study on Tlr4 gene regulation in vitro and in vivo confirms that the promoters in mouse and human are indeed different, and these differences affect Tlr4 regulation on both the transcriptional and the translational levels. Fig. 7 summarizes our current understanding of TLR4 promoter structures and regulatory mechanisms in mouse and human that are discussed further below.

In terms of translation efficiency, we identified two mouse-specific out-of-frame ATGs that markedly influenced luciferase activity in reporter gene assays. When mutated in mouse constructs, Tlr4 promoter activity increased 2-fold and, when introduced into human constructs, their activity markedly dropped. Previous studies showed that open reading frames created by upstream ATGs (upstream open reading frames) can control gene expression by translational mechanisms. During protein synthesis, ribosomes initiate translation of upstream open reading frames at upstream ATGs and often fail to reinitiate at the actual coding ATG. Studies on the well characterized oncogene HER2 (32, 34) indicate that proteins binding a translational de-repressing element of the 3′-untranslated region may additionally influence the ability of ribosomes to translate the coding open reading frame. So far, it is unclear to which extent the observed upstream, out-of-frame ATGs influence TLR4 translation in primary cells. However, upstream ATGs are a common feature of human and murine TLR5′-untranslated regions and affect roughly 80% of TLR genes. Studies using Tlr4 transgenic mice indicate that responsiveness to TLR agonists likely depends on TLR expression levels. Upstream ATGs in TLR genes may therefore serve to limit the number of available receptors in the presence of continuous mRNA expression.

Previous transfection-based studies suggested major differences in Tlr4 promoter structures in mouse and human macrophages. Human macrophages were found to require two proximal PU.1 binding elements for Tlr4 reporter activity (10), whereas in murine macrophages, a different upstream PU.1 element was necessary to drive Tlr4 reporter gene activity (16, 17). However, evidence from ChIP assays, genomic footprinting, gel shifts, and TSS analyses from our current study strongly suggests that TLR4 transcription in murine and human macrophages initiates from the same conserved promoter region, and PU.1 occupies the same conserved sites in both species.

A major functional difference between human and murine Tlr4 genes is an altered promoter usage in non-myeloid cells.
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Murine non-myeloid cells such as intestinal epithelium cells exclusively utilize an alternative distal promoter, whereas human epithelial cells use both the “myeloid” proximal promoter, and also, albeit to a lesser extent, the distal promoter. In line with earlier studies, we confirmed the importance of an element that was originally identified as an AP-1 consensus motif (15, 17, 35). Our studies show that this region actually comprises a conserved E-box element that is required for reporter gene activity in fibroblasts. At this site, USF factors are the dominant binding factors in gel shift experiments. However, co-expression studies using expression plasmids for activating or dominant-negative forms of E-box binding factors argue for a more important role of microphthalmia TFE factors in activating the distal promoter. Additional studies are required to identify the factors that are relevant for Tlr4 expression in individual non-myeloid cell types in vivo.

Interestingly, the distal promoter appeared to be less important in humans. Although the proximal promoter was strictly myeloid in humans. Although the proximal promoter was strictly

servers affect transcription also in non-myeloid cells. Transfection analyses suggest that the human promoter contains a human-specific transcription factor in gel shift experiments. However, co-expression studies using expression plasmids for activating or dominant-negative forms of E-box binding factors argue for a more important role of microphthalmia TFE factors in activating the distal promoter. Additional studies are required to identify the factors that are relevant for Tlr4 expression in individual non-myeloid cell types in vivo.

Our experiments suggest that in mice, PU.1 is involved in directing the transcription initiation complex to the proximal promoter. In the murine PU.1 inducible progenitor cell line PUER (18), the distal promoter was utilized for Tlr4 transcription in the absence of PU.1, and the start site was shifted to the downstream myeloid promoter within 15 min of PU.1 activation. This rapid PU.1-dependent change in TSS adds direct evidence to previous experiments demonstrating that the activation domain of transcription factor PU.1 binds the transcription factor TFIIID in vitro and that an array of Ets/PU.1 motifs is sufficient to drive reporter gene expression in macrophages (8). This property of PU.1 may be of particular importance in the absence of elements such as TATA and GC boxes or INR elements that typically stabilize the initiation complex. This is consistent with recently published experiments by one of us (9) that demonstrated an absolute requirement for PU.1 with respect to the recruitment of the basal transcription machinery at the myeloid-specific c-fms promoter. However, the Tlr4 locus differs from c-fms in that it employs a mechanism that utilizes different combinations of transcription factors and promoter elements to initiate expression in myeloid as well as non-myeloid cells. Our data here show that this feature appears to be under evolutionary pressure. Although both mice and humans use two transcriptional start sites, mice have evolved to switch promoter elements in myeloid cells that express PU.1.

This feature may have evolved to accommodate a wider tissue distribution of Tlr4 expression in mice. It will be interesting to see in more molecular detail how these species-specific differences affect Tlr4 expression, in particular in different non-myeloid cell types. Our current study provides the tools and foundations for further studies on the regulation of an important component of our innate immune system.

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