Three Promoters Regulate Tissue- and Cell Type-specific Expression of Murine Interleukin-1 Receptor Type I*

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The type 1 interleukin-1 receptor (IL-1R1) mediates diverse functions of interleukin-1 (IL-1) in the nervous, immune, and neuroendocrine systems. It has been suggested previously that the versatile functions of IL-1 may in part be conferred by the multiple promoters of IL-1R1 that have been identified for the human IL-1R1 gene. Promoters for murine IL-1R1 (mIL-1R1) gene have not been studied in detail. We performed 5′-rapid amplification of cDNA ends to determine the transcription start sites (TSS) in mIL-1R1, using mRNAs derived from 24 different tissues. The results revealed three putative TSSs of mIL-1R1. Three full-length cDNAs containing these distinct TSSs were recovered in screens of cloned cDNA libraries. Translation of these cDNAs produced IL-1R1 proteins that were verified by Western blot analysis. IL-1 stimulation of the individual IL-1R1 proteins resulted in the activation of NF-κB. Promoter-reporter assay for genomic DNA sequences immediately upstream of the three TSSs validated that the sequences possess promoter activity in a cell type-specific manner. These promoters are termed P1, P2, and P3 of the mIL-1R1, in 5′ to 3′ order. Quantitative PCR analysis of P1-, P2-, and P3-specific mIL-1R1 mRNAs showed that there is tissue-specific distribution of these mRNAs in vivo, and there are distinct patterns of P1, P2, and P3 mRNA expression in different cell lines. In the brain, P3 mRNA is expressed preferentially in the dentate gyrus. Further, glucocorticoids differentially regulate these promoters in a cell type-specific manner. Together, these results suggest that the different IL-1R1 promoters contribute to the discrete and diverse actions of IL-1.

Interleukin-1 is a pro-inflammatory cytokine that plays multiple roles in the immune, nervous, and neuroendocrine systems (1). The biological activity of IL-1β is mediated by distinct IL-1 receptors. A receptor for both IL-1α and IL-1β was first cloned from mouse T cells (2). This receptor is the type I IL-1 receptor (IL-1R1). In B cells (3) and monocytes (4), a second type of IL-1 receptor was discovered. This receptor was later cloned and named the type II IL-1 receptor (IL-1R2) (5). IL-1R2 was later shown to serve as a decoy receptor for IL-1, negatively regulating the activity for IL-1 (6). In some cells, IL-1R1 is exquisitely sensitive to IL-1 stimulation, requiring only 10 molecules/cell to mediate potent stimulatory effects (6). In a B-cell cell line 70Z/3, IL-1R2 but not IL-1R1 could be detected, yet IL-1 signaling in these cells is mediated exclusively by IL-1R1, suggesting that trace amounts of IL-1R1 mediate the effect of IL-1 (7) despite the presence of large quantities of IL-1R2. In general, it is widely accepted, with rare exceptions (8), that IL-1R1 is the receptor that mediates the biological effects of IL-1.

The promoters for the IL-1R1 gene have not been studied in depth. Ye et al. (9) were the first to identify multiple transcription start sites (TSS) in the human IL-1R1 gene by primer extension analysis. They later provided evidence that human IL-1R1 gene expression may be controlled by three distinct promoters (10). Clinically, a PstI polymorphism near the human IL-1R1 promoter 2 (P2) was found to have significant association with insulin-dependent diabetes mellitus (11). A polymorphism within Exon 1B has also been associated with protective effects against endometriosis development (12). Exon 1B expression is driven specifically by the P2. Therefore, the here-tofore under-characterized IL-1R1 promoters may play critical roles in IL-1 biology.

We chose to characterize the murine IL-1R1 (mIL-1R1) promoters to permit in-depth analysis of their structure and function in vivo and in vitro. Thus far, only two putative TSSs for mIL-1R1 mRNA can be found in the GenBank™, but no promoter analysis has been reported. In this study, we identified and characterized the mIL-1R1 promoters and determined that they contribute to the differential expression and regulation of mIL-1R1 mRNA.

**EXPERIMENTAL PROCEDURES**

Reagents and Cell Lines—Luciferase reporter vector pGL4.10 was obtained from Promega (Madison, WI). Lipofectamine LTX with Plus reagents and SteadyHIT HTS for luciferase assay were purchased from PerkinElmer Life Sciences. Rabbit polyclonal antibody against mIL-1R1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Power SYBR Green PCR

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Master Mix was purchased from Applied Biosystems (Foster City, CA), RAW 264.7 (macrophage), D10.G4.1 (Th2 cell), Neuro-2a (neuroblast cell), SVEC4–10 (peripheral endothelial cell), C8-DIA (type 1 astrocyte), C8-D30 (type 3 astrocyte), C8-S (type 2 astrocyte), bEnd.3 (brain endothelial cell), and LADMAC (macrophage progenitor) were purchased from ATCC (Manassas, VA). These cell lines were maintained according to the instructions of the ATCC protocols.

5’-Rapid Amplification of cDNA Ends—The Mouse Sure-RACE kit (OriGene, Rockville, MD) containing PCR-ready mouse cDNAs (generated from 24 different tissues) was used. The 5’ end of mRNA in this kit has been modified to contain 5’ adaptor sequences (ADP) to facilitate PCR amplification of the 5’ ends of mRNAs. Two gene-specific primers (GSP) for mIL-1R1 were designed from the published mIL-1R1 mRNA sequence in GenBankTM (accession number M20658). The sequence for GSP1 is 5’-TACGTCAATCTCAGCAAGCAGCAGAG, and the sequence for GSP2 is 5’-AGGAGAGGCACCATGAGAATAG. These primers are cloned in Exon 2 of the published mouse IL-1R1 mRNA sequence, after the putative start codon.

Nested PCR was performed with a pair of outer anchor primers, ADP1 and GSP1, followed by a pair of inner anchor primers, ADP2 and GSP2. A graphic depiction of the RACE design is shown in Fig. 1A. The PCR products were separated by electrophoresis using a Bioanalyzer (Agilent, Santa Clara, CA). For the RACE-PCR ampicons with a single major band, the products were directly cloned into the PCR 2.1-TOPO vector by TOPO TA cloning (Invitrogen). For the RACE-PCR amplicons containing multiple bands, all of the visible bands were resolved by 10% agarose gel electrophoresis and purified. The isolated bands were subsequently cloned into the PCR 2.1-TOPO vector. The cloned cDNAs were sequenced by an automatic sequencer (Plant-Microbe Genomics Facility at Ohio State University). The sequence data were aligned to the mouse genomic data base of the National Center for Biotechnology Information.

mRNA Library Screening—Sequence data from the RACE assay revealed three different alternative first exons of mIL-1R1. We annotated these findings in the context of known genomic structure of mIL-1R1 in Fig. 1C and designated these alternative first exons as Exon1A, Exon1B, and Exon1C. We then designed PCR primers to target these exons. The primer pairs for Exon1A, Exon1B, and Exon1C are 5’-GATGTCAT-CAGAGTCTCCAGTG/5’-CATTCTGCTCTGATAATCCTG, 5’-CGTGGTTAGTTGGAATCAGTC/5’-CATTCTGGTGAATCCTG, and 5’-AGGACACTGACATTTCCCTGC/5’-CATTCTGCTGATAATCCTG, respectively. These primers were sent to OriGene for PCR-screening using the OriGene Rapid-Screen Master plates containing full-length mRNA libraries generated from adult brain (MAB-1001), embryonic day 19 (MEA-1001), embryonic day 12.5 (MEB-1001), adult liver (MLI-1001), thymus (MTM-1001), and adult testis tissues (MTS-1001). Screening from a total number of three million clones yielded three full-length cDNAs containing Exon1A, Exon1B, or Exon1C. These cDNAs were cloned into the expression vector pCMV6-XL4. These clones were sequenced to verify that the Exon1A, Exon1B, and Exon1C indeed exist in different full-length IL-1R1 mRNA species. Because the three exon 1 alternatives suggest that there are three different promoters for the mIL-1R1, we designated these clones as P1-, P2-, and P3-cDNA clones.

Western Blot—To study protein products of these three different mIL-1R1 cDNA clones, the cDNAs were transfected into Neuro-2a cells. Transfection was performed with Lipofectamine LTX and Plus reagents (Invitrogen) according to the Invitrogen protocol. Twenty-four h after the transfection, the cells were harvested and washed twice with cold phosphate-buffered saline, and 2 x 10⁶ cells were resuspended in 250 μl of phosphate-buffered saline. Then 50 μl of 6 x loading buffer (0.35 M Tris-HCl, pH 6.8, 10.28% SDS, 36% glycerol, 0.6 M diethiothreitol, and 0.01% phenol red) was added. The samples were briefly sonicated and heated to 95 °C for 5 min in preparation for Western blotting. Twenty-microliter solution from each sample was resolved by SDS-PAGE electrophoresis and transferred to polyvinylidene difluoride membranes. The blot was then blocked with 10% milk in TBS and probed with a rabbit polyclonal antibody against mouse IL-1R1 (sc-689;Santa Cruz Biotechnology) in 1% milk TBS-T followed by the IRDye 680 Goat anti-rabbit antibody (LI-COR Inc. Lincoln, NE). The signal was detected using the LI-COR infrared imaging system.

NF-κB Reporter Assay—To study the function of proteins translated from the P1-, P2-, and P3-initiated mRNAs of mIL-1R1, we tested the ability of these proteins to mediate IL-1-induced NF-κB activation, a well known IL-1 activity. The MHC-3xkB-Luc luciferase reporter, an NF-κB reporter construct, was obtained from Dr. Denis Guttridge (Ohio State University, Columbus, Ohio). In a 24-well culture plate, P1-, P2-, or P3-cDNA (0.1 μg) was co-transfected with MHC-3xkB-Luc (0.4 μg) in a molar ratio of 1:5 into the Neuro-2a cells. Twenty-four h after the transfection, IL-1α was added to each well (the resulting concentration of IL-1α was 100 pg/ml), and the cells were incubated for additional 6 h. Luciferase activity was measured by the SteadyLite HTS assay system and a VICTOR3 Multi-Label Reader (both from PerkinElmer Life Sciences).

Promoter-Reporter Constructs—After aligning the sequences of P1-, P2-, and P3-mRNA to the genomic mIL-1R1 sequence, three putative transcription start sites (TSS) were identified. To determine promoter activities upstream of these TSSs, 500–800-bp (core promoter region, CP) and 2-kb (long promoter region, LP) upstream genomic sequences were PCR-amplified. The following primer pairs were used: 5’-TGTGACACGC-ATTCTGAGGTTG/5’-TCAAGTGAGCCAGCACGAGA/CP1, 5’-AACTTCCATCGGAAGATACC/5’-GTCAGAATCGCGATCAT/CP1 (LP1), 5’-TGATTCCTTGGCACCCT-CTGC/5’-ACCTTCTGAGCAGCAGACGCTG/CP2, 5’-CAGCAATGTCATGCTGAAGCGAAG/CP3 (LP2); 5’-CACCAATGTCATGCTGAAGCGAAG/CP3 (LP2); and 5’-TCAGCTCTCATGGCCCAAGA/CP3 (LP3). Each sample was resolved by SDS-PAGE electrophoresis and transferred to polyvinylidene difluoride membranes. The blot was then blocked with 10% milk in TBS and probed with an IRDye 680 goat anti-rabbit antibody (LI-COR Inc. Lincoln, NE). The signal was detected using the LI-COR infrared imaging system.
construct downstream of the luciferase reporter sequence to generate the promoter-reporter-enhancer constructs (a graphic depiction of the promoter-reporter constructs is shown in Fig. 3, A and B).

Promoter-Reporter Assay—Promoter-reporter constructs were transfected into the Neuro-2a (neuron), RAW 264.7 (macrophage), and SVEC4–10 (endothelial cell) cells using the Lipofectamine LTX and PLUS reagents as described above. These cells were always grown to be 50–80% confluent at the time of transfection. Transfected cells were incubated at 37 °C in a CO₂ incubator for 24 h before the analysis of luciferase activity using the same method described above.

Analysis of P1-, P2-, and P3-mRNA Expression Patterns in Various Cell Lines and in Different Tissues—Total RNA was isolated from nine different cultured cells (RAW 264.7, D10.G4.1, Neuro-2a, SVEC4–10, C8-DIA, C8-D30, C8-S, bEnd.3, and LADMAC) and nine major tissues (heart, spleen, lung, pituitary, liver, kidney, adrenal gland, thymus, and testis) followed by the generation of cDNAs by reverse transcription. The mRNA levels were analyzed by SYBR Green-based quantitative PCR. The following primer sets for quantitative PCR analysis of P1-, P2-, and P3-mRNA transcripts were used. The forward primers for P1-, P2-, and P3-mRNAs were 5′-GATGT-CATCAGAGTCCCAAGT, 5′-CTGTCTGAATTGTGAAA- CATCG, and 5′-AGGACACTGAGACATTCCGG, respectively. These different primers reside in Exon1A, Exon1B, and Exon1C, respectively. A common reverse primer 5′-TGAGGATAAGGAGGACACTTGG was used. This sequence is complementary to a sequence in the Exon 3 of the mIL-1R1 mRNA. This common reverse primer was used because P1-, P2-, and P3-mRNAs share this common downstream sequence, and Exon 3 is separated from all the alternative exons 1 by Intron 2. This design minimizes the possibility that genomic DNA of P1, P2, and P3 activity by glucocorticoids, Neuro-2a, SVEC4–10, and RAW246.7 cells were incubated with varying concentrations of dexamethasone (Dex) or vehicle overnight. Changes in P1-, P2-, and P3-IL-1R1 mRNA levels were analyzed by quantitative RT-PCR as described above. To study the effect of glucocorticoids in vivo, the animals were injected intraperitoneally with Dex (5 mg/kg). Tissue mRNA was extracted 2 h after the injection, and levels of promoter-specific IL-1R1 mRNA were analyzed by quantitative RT-PCR. The responsiveness of SVEC4–10 and RAW246.7 cells to IL-1 stimulation after these cells were treated with Dex or vehicle was compared. Briefly, the cells were incubated with 10⁻⁶ M of Dex or vehicle overnight and then washed with culture medium three times before IL-1 (final concentration of IL-1α in the cultures was 1 ng/ml) was added to the cultures. RNA was extracted from these cultured cells 2 h later and analyzed for the expression of cyclooxygenase 2 (COX-2) mRNA levels using quantitative RT-PCR as we described previously (14).

Statistical Analysis—The data are presented as the means ± S.E. Variations in mRNA levels were evaluated by one-way analysis of variance followed by post-hoc analysis (Tukey test). p < 0.05 is considered statistically significant.

RESULTS

Fig. 1B shows results of electrophoresis of 5′-RACE PCR products from various mouse tissues listed in the Fig. 1 caption. Multiple band patterns were obvious when PCR products from different tissues were compared. In some tissues, e.g. brain, stomach, and skin, multiple bands (indicated by asterisks) were
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The sequences are aligned to the latest version of NT_039170.7.

TABLE 1
Sequence analysis of murine IL-1R1 TSS

| Exon I Start site | Exon I End | Exon II start site | Exon II End | Transcripts in different tissues |
|-------------------|------------|--------------------|------------|----------------------------------|
| 17808499          | 17808655  | 17865729           | 6, 24      |
| 17808523          | 17808803  | 17865729           | 1          |
| 17808523          | 17808655  | 17865729           | 6, 5       |
| 17808558          | 17808655  | 17865729           | 1, 11, 23  |
| 17808576          | 17808803  | 17865729           | NM_008362  |
| 17808627          | 17808803  | 17865729           | 6, 20      |
| 17808670          | 17808803  | 17865729           | BC109135   |
| 17850082          | 17850378  | 17865729           | AK054137   |
| 17850164          | 17850378  | 17865729           | AK048550   |
| 17850309          | 17850378  | 17865729           | 1, 11, 6, 23 |
| 17863552          | 17865792  | No intron gap      | 24         |

generated by the RACE-PCR. These bands were isolated, cloned into the TOPO 2.1 vector, and sequenced. In other tissue, e.g. thymus and muscle, only one major band was generated. PCR products from these tissues were directly cloned into TOPO vector and sequenced.

The sequences generated from the RACE clones were aligned to the genomic DNA sequence of mL-1R1 by two-sequence BLAST. The results are summarized in Table 1. Only the unique sequences are listed. Sequences found in the existing data base with GenBank accession numbers as references were also included. Many TSS were identified in the different tissues. Three major groups of TSS in IL-1R1 were found. The first group of TSS (the numbers for the start sites are in regular font in Table 1) spans 271 bp, aligning to nucleotide positions 17808499–17808670 of the reference contig sequence NT_039170.7. This group of TSSs is close to two published mRNA start sites (accession numbers NM_008362 and BC109135), although some of the TSSs discovered in the present studies are further upstream, whereas others are downstream to the published TSSs. The second group of TSSs (the numbers for the start sites are in bold type in Table 1) was found clustered close to the position of 17850082 of the NT_039170.7 sequence. These TSSs are ~41 kb downstream of the first group of TSS sites. These TSSs are slightly downstream of the published TSSs at this locus (accession numbers AK054357 and AK048550). The third type of TSS was cloned from the involuting breast tissue (sample 24 in the RACE kit). This rare TSS (italic in Table 1) is located immediately in front of the second exon of the IL-1R1 gene. In fact, the mRNA sequence of this subspecies of IL-1R1 mRNA lacks the entire first intron defined in the GenBank database. No reported TSS matched this TSS.

To verify that these three groups of TSSs, resulting in three different Exons 1 (designated as Exon 1A, Exon 1B, and Exon 1C), are found in full-length mL-1R1 mRNAs, PCR primers were designed to screen the full-length mRNA libraries from OriGene (three million randomly cloned full-length cDNA from six different tissues). We obtained three clones after the screening that confirmed the existence of three alternative Exons 1 in mL-1R1 mRNA. Because these three alternative Exons 1 suggest three different promoters, we designated these putative promoters as the P1, P2, and P3 of the mL-1R1, in the 5’ to 3’ order. The mRNAs driven by these promoters are denoted as the P1-, P2-, and P3-mRNA. The positions of the IL-1R1 promoters are annotated in Fig. 1C in the context of known genomic structure of the mL-1R1 gene. The complete P1-, P2-, and P3-mRNA sequences are presented in the supplemental Fig. S1.

Simplified exon structure diagrams for P1-, P2-, and P3-mRNAs are shown in Fig. 2A. The only difference found among these three mRNAs is at the 5’ end. Exon 1A, Exon 1B, and Exon 1C are distinct. Analysis of open reading frames showed that whereas the predicted start codon for P2-mRNA resides in exon 2, the predicted start codon for P1-mRNA resides in Exon 1A, resulting in an addition of two more amino acids at the N terminus. The Exon 1C is the longest exon, shifting the predicted open reading frame significantly toward the 5’ end and resulting in the addition of 43 additional amino acids at the N terminus.

P1-, P2-, and P3-cDNA were transfected into the Neuro-2a cells. Expression of these cDNAs was examined by Western blotting using an antibody targeted at the C terminus of the mL-1R1. Fig. 2B shows that IL-1R1 protein was not detected in Neuro-2a cells transfected with an empty vector. IL-1R1 protein was found in Neuro-2a cells transfected with either P1-, P2-, or P3-cDNA. No difference in molecular weight was found among the detected IL-1R1 proteins extracted from P1-, P2-,
and P3-cDNA transfected cells. Two bands were noted in the Western blot. This band pattern is consistent with that published by the manufacturer of the antibody used in this study; this antibody probably detects two IL-1R1 isoforms resulting from different glycosylation of the IL-1R1 protein.

Fig. 2C shows results from the NF-κB reporter assay. Neuro-2a cells were co-transfected with P1-, P2-, or P3-cDNA together with the NF-κB reporter (NFR). The cells were then stimulated with 100 pg/ml of IL-1α. In a control experiment, the cells were transfected with NFR together with the empty vector for the IL-1 cDNAs (V). IL-1 stimulation did not induce NF-κB activation in cells transfected with the empty vector. In co-transfected cells that contained both IL-1 cDNA and the NFR, IL-1 stimulation resulted in significant NF-κB activation as indicated by the increased luciferase activity. No difference was detected among P1-, P2-, and P3-cDNA transfected cells.

Fig. 3 shows promoter activity detected by the promoter-reporter assay. 2-kb DNA sequences immediately upstream of the TSSs found in the P1-, P2-, and P3-mRNAs were studied. These sequences were designated as the long promoters. Hence they are denoted as LP1, LP2, and LP3. The 3’ end of the long promoters also included 100–150 bp of the 5’-untranslated region. We also investigated the promoter activity in shorter DNA sequences (~500 bp) near the TSSs. These sequences are called core promoters and are hence designated as CP1, CP2, and CP3. The positional relationship between LP1 and CP1,
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LP2 and CP2, and LP3 and CP3 is shown in Fig. 3A. These promoters were studied in the peripheral endothelial (SVEC4–10), macrophage (RAW264.7), and neuronal (Neuro-2a) cell lines. Fig. 3B shows the summary of promoter activity determined in this study. LP2 exhibits the strongest activity in all three cell lines, whereas LP1 and LP3 showed detectable but small promoter activities. The activity of the LPs was also tested when a SVE sequence was added. LP1 activity was increased by the addition of SVE in all three cell lines (LP1 versus LP1+SVE, p < 0.05). SVE increased LP2 and LP3 activity in both the endothelial and macrophase cell lines but did not increase LP2 activity in the neuronal cell line. CP1 showed stronger promoter activity than LP1 in both the endothelial cells and macrophase cells, but CP1 was not active in the neuronal cell line. CP2 showed stronger promoter activity than LP2 in all three tested cell lines. CP3 activity was not greater than LP3 activity in any of the cell lines tested (CP3 versus LP3, p > 0.05). Because the promoter activity of CP3 was weak (the activity was barely stronger than the promoter-less construct), we tested the promoter activity of CP3+SVE. Fig. 3D shows that SVE increased CP3 activity in both endothelial cells and macrophase cells but not in the neuronal cells.

Fig. 4A shows patterns of P1- and P2-mRNA expression in various cell lines. P2 mRNA was the dominantly expressed IL-1R1 in D10.G4.1, LADMAC, and SVEC4.10 cells. P1-mRNA was the dominantly expressed IL-1R1 in b.End3 cells. Very little IL-1R1 mRNA was detected in C8-DIA, C8-S, C8-D30, RAW264.7, and Neuro-2a cells. P3-mRNA was expressed at very low levels in all of the cells tested (data not shown). Fig. 4B shows the tissue distribution of P1-, P2-, and P3-IL-1R1. In the liver, P1 is the dominantly expressed IL-1R1. In the lung, spleen, heart, thymus, testis, adrenal gland, and kidney, P2 is the dominantly expressed IL-1R1 mRNA. P3-mRNA was clearly detected in many tissues. In the liver, P3-mRNA was expressed at a higher level than P2-mRNA.

Among the three tissues tested in the brain, the highest P1-mRNA expression was found in the striatum, whereas the highest P2-mRNA expression was found in the dentate gyrus. P3-mRNA was preferentially expressed in the dentate gyrus (Fig. 4C).

Because the P3-mRNA sequence is significantly different from the P1- and P2-mRNA sequence at the 5' end, we further investigated the in situ hybridization histochemistry labeling patterns of the P3-mRNA. Fig. 4D shows that P3-mRNA was only detected in the dentate gyrus from both coronal and sagittal sections of the brain.

The core promoter sequences of the three IL-1R1 promoters were subjected to a web-based transcription factor-binding site analysis. The results are shown in supplemental Fig. S2. A diagrammatic presentation of the putative transcription factor-binding sites on these promoter sequences is shown in Fig. 5A. A glucocorticoid receptor (GR)-binding site was noted only in CP1, not in CP2 and CP3, whereas an NF-κB-binding site was found only in CP2, and a CREB site was found only in CP3. An AP1 site was found in both CP2 and CP3.

We tested whether activation of GR selectively modulated these promoters. Fig. 5B shows that in RAW264.7 SVEC4–10 and Neuro-2a cells, adding glucocorticoid agonist Dex up-regulated IL-1R1 expression only in the RAW cells. The increased IL-1R1 was exclusively P1-IL-1R1. Fig. 5C shows that the Dex-induced P1-mRNA expression was dose-dependent, and the threshold concentration for Dex-induced P1-IL-1R1 expression was 10^{-8} M. Fig. 5D shows that injection of Dex in vivo induced IL-1R1 in the liver. Interestingly, besides P1-IL-1R1, P3-IL-1R1 was also up-regulated.
Fig. 6 shows induction of COX-2 expression by IL-1/H9251 (1 ng/ml) in RAW267.7 and SVEC4–10 cells after these cells were pretreated with either Dex (10⁻⁶ M) or vehicle. The data are presented as the relative levels of COX-2 mRNA normalized against the mRNA levels of the housekeeping gene G3PDH. Dex treatment significantly increased IL-1-induced COX-2 expression in RAW 267.7 cells but not in SVEC4–10 cells.

DISCUSSION

The results of this study revealed three functionally relevant clusters of TSSs, each with a unique promoter, in the murine IL-1R1 gene. Determination of the TSSs for IL-1R1 is technically difficult because IL-1R1 is typically expressed at very low levels. Previous studies have used large amounts of mRNA isolated from individual cell lines to determine the IL-1R1 TSS by primer extension assay (9). Using RNA generated from multiple tissues and the more sensitive 5’-RACE method, we found many novel TSS sites that are not in the current GenBank™ data base.

Both primer extension and 5’-RAGE may produce artifactual putative TSSs by identifying 5’ truncated RNA. However, all of the TSSs associated with P1 and P2 promoters were found in multiple tissues in multiple clones (Table 1), suggesting that the results were unlikely to be the consequence of faulty RACE-PCR amplification of randomly truncated RNAs. In the case of P1-IL-1R1, we actually found TSSs further upstream from the reported TSSs generated from full-length mRNA libraries (in Table 1, BC109135 was a sequence reported by the RIKEN full-length enriched library). Therefore, these newly identified TSSs are likely to be the true TSSs. Inspection of the putative promoter sequences upstream from these TSSs showed no TATA box consensus sequence. This is consistent with previous reports that IL-1R1 uses TATA-less promoters (10). Other studies have demonstrated that TATA-less promoters often start transcription from several TSSs downstream of the promoter (15). Therefore, the clusters of TSSs found downstream of the putative P1 and P2 promoters might reflect this characteristic of this type of promoter. The TSS for P3 was only found once in the RACE assay. To exclude the possibility that this result is a PCR artifact, we screened the full-length mRNA libraries from OriGene to determine whether this TSS existed in a full-length cDNA clone. A clone was obtained from the screening process that indeed contained the P3-TSS.
Previous studies showed that the translated IL-1R1 protein contains a signal peptide, three immunoglobulin-like extracellular domains, a transmembrane domain, and an intracellular domain in the N-to-C terminus order (2). The signal peptide is digested to generate the mature IL-1R1. The mRNA sequences for the P1- and P2-IL-1R1 discovered in the present study do not predict a translated IL-1R1 protein that is different from that found in previous studies. The mRNA sequence for the P3-IL-1R1, however, predicts a new open reading frame that adds 43 amino acids to the N terminus of the IL-1R1. We found all three IL-1R1 mRNAs translated into IL-1R1 proteins, although smaller amounts of IL-1 protein appear to be made from the P3-IL-1R1 (Fig. 2B). It is possible that the long 5’-untranslated region in the P3-mRNA reduces translation efficiency. No differences in the molecular weights of these IL-1R1 proteins were noted. It is likely that after digestion of the signal peptide, which is translated from the common Exon 2, identical mature IL-1R1 proteins are produced from all three mRNAs. It is possible that small differences in molecular weights between these IL-1R1 proteins were not detected by the Western analysis. Nonetheless, when the functions of these IL-1R1 proteins were examined, P1-, P2-, and P3-IL-1R1 were all equally able to mediate IL-1-induced NF-κB activation. Therefore, these different IL-1R1 promoters are likely to produce the same mature IL-1R1 protein as that identified in previous studies. However, the possibility remains open that subtle changes in receptor function are produced by transcription-initiated translational modifications.

The TSS sites identified for the P1 and P2 promoters are very close to those from previously submitted sequences. Therefore the positions of P1 and P2 can also be computationally predicted from the existing data base. Experimental verification of promoter activity for these potential promoters, however, has not been reported. We show in this study that both the 2-kb sequence (LP1 and LP2) and the 500–800-bp sequences (CP1 and CP2) in transiently transfected promoter-reporter constructs exhibit true promoter activity. Therefore, the essential promoter regions for the P1 and P2 promoters reside in the CP1 and CP2 sequences. In addition, promoter activity of both LP1 and LP2 can be modulated by the SVE. Interestingly, SVE enhanced the activities of both LP1 and LP2 over 20-fold in the macrophage cell line, RAW264.7, but SVE did not significantly increase LP1 activity and reduced LP2 activity in the Neuro-2a cells. In the endothelial cell line, SVEC4–10, SVE increased LP1 activity by 2-fold and LP2 by 4.6-fold. These results suggest that the activities of the promoters are modulated differently in different cell types. The completely novel P3 promoter showed weak promoter activity. Both
LP3 and CP3 activities were enhanced by SVE in the macrophage and the endothelial cell lines, but SVE did not increase LP3 and CP3 activities in the Neuro-2a cells. These results show that the newly discovered P3 promoter also exhibits true promoter activity, and its activity is modulated in a cell type-specific manner.

Although the promoter-reporter assay was very informative in terms of demonstrating whether the predicted promoter sequences can truly drive the transcription of downstream sequences, this assay does not assess the activity of the mIL-1R1 promoters in their normal chromosomal/chromatin context. We analyzed the levels of P1-, P2-, and P3-mRNA to determine the activities of the endogenous activities of P1, P2, and P3. In nine cell lines tested, very little P3-driven expression was found, suggesting that this promoter is repressed in most cell types. P2 was the dominant driver of IL-1R1 mRNA expression in all of the cell lines tested but one. These results are consistent with those obtained in the promoter-reporter assay, suggesting that P2 may be the dominant promoter in most cells. It is also interesting to note that whereas P2 mRNA was dominantly expressed in the peripheral endothelial cell line SVEC4–10, P1 mRNA was dominantly expressed in a brain endothelial cell line bEnd.3. Thus, subtypes of endothelial cells in different tissues may use different IL-1R1 promoters to control IL-1R1 expression. In vivo, P2-dominant IL-1R1 expression can be shown in most tissues (Fig. 4B) except in liver and pituitary. The pattern of liver IL-1R1 expression is striking because over 90% of IL-1R1 was P1-IL-1R1. These results suggest that P2 promoter may be highly repressed in the liver. In addition, P3-IL-1 was detectable in many tissues at low levels. Thus, P3 may be used by rare cell types in vivo.

IL-1 exhibits complex function in brain. IL-1 is known to affect neuroimmune communication via brain endothelial cells (16, 17), modulate brain inflammation via astrocytes (18) and microglia (19), and influence learning and memory via hippocampal neurons (20, 21). The presence of IL-1 receptors in the brain was first demonstrated by binding studies that showed that IL-1 binds to specific structures in the brain including dentate gyrus granule cells, choroid plexus, and brain vasculature (22). In the present study, P1-mRNA was found to be the dominant IL-1R1 expressed in the striatum (Fig. 4C), where previous studies found that IL-1R1 is expressed mainly in endothelial cells (22). This is consistent with the finding in Fig. 4A that P1-mRNA was the dominantly expressed IL-1R1 in the brain endothelial cell line, bEnd.3. In dentate gyrus and in choroid plexus, P2-mRNA is higher than P1-mRNA. In the dentate gyrus, the primary cell type expressing full-length IL-1R1 mRNA (20) are granule neurons. In the choroid plexus, the primary expressing cell type may be choroidal epithelial cells. It is thus possible that in these structures, IL-1R1 is expressed mainly by cell types that are different from the IL-1R1-expressing cells in the striatum, suggesting that the P1 promoter drives expression in endothelial cells and the P2 promoter drives expression in neuronal/epithelial cells. When the P3-mRNA level is compared among the three tested brain structures, it is significantly higher in the dentate gyrus than in the striatum and choroid plexus (Fig. 4C), suggesting that P3 mRNA is preferentially expressed in the dentate gyrus, notably in neurons (Fig. 4D). The 5’ end of the P3 mRNA is significantly different from the P1- and P2-mRNA (Fig. 2A). This difference may have contributed to the lack of detection of IL-1R1 mRNA in the dentate gyrus in the study of rat brain by Ericsson et al. (23), although its detection in mouse brain is robust (20). Future work may reveal that mouse and rat have different P3 mRNA promoter activities in the dentate gyrus.

Diverse regulation patterns of IL-1R1 have been observed in the literature. For example, IL-1R1 mRNA was found to be down-regulated by IL-1 in brain endothelial cells, but not in neurons (23). In hepatocytes, IL-1 was found to up-regulate the expression of IL-1R1 (24). In the mouse brain, LPS treatment was able to reduce the expression of IL-1R in the dentate gyrus but did not change IL-1R expression in the pituitary (25). On the other hand, glucocorticoid was found to modulate the density of ligand binding to the pituitary IL-1R but not neuronal IL-1R (25). Surprisingly, although glucocorticoid usually antagonizes the immunological effects of IL-1, several reports found glucocorticoid up-regulated IL-1R binding (26) and IL-1R (27, 28) expression in macrophages and neutrophils. These findings suggest that depending on tissue and cell type, the same signaling molecule could cause different, even opposite, regulatory responses of IL-1R1 gene expression. The discoveries in the present study provide a molecular mechanism to explain the observed diversity in the regulation of IL-1R1 expression. Fig. 5A shows unique transcription factor-binding sites in CP1, CP2, and CP3. Therefore, different transcription factors will affect different promoters of IL-1R1, resulting in differential regulation of IL-1R1 expression. Fig. 5B shows a striking example that IL-1R1 is selectively up-regulated in the macrophage cell line by Dex, and the increased expression of IL-1R1 was exclusively P1-IL-1R1. This is consistent with the fact that a GR-binding site is only found in CP1, not in CP2 or CP3. Interestingly, although P1 mRNA can also be found in the endothelial cell line SVEC4–10, P1-IL-1R1 was not up-regulated in this cell line. Therefore, IL-1R1 expression is modulated by transcription factors that interacted with the specific promoter sequences that contained corresponding binding sites and fac-
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tors that restricted cell type-specific activation of the specific promoters. In vivo, IL-1R1 mRNA was found to be significantly up-regulated by Dex treatment in the liver. Again, this is consistent with the finding that liver IL-1R1 is primarily P1-IL-1R1. Interestingly, P3-IL-1R1 was equally up-regulated by the Dex treatment in the liver. The lack of GR-binding site in CP3 and the fact that P3-IL-1R1 was not up-regulated in other tissues and in the tested cell lines suggest that P3-IL-1R1 in the liver may be up-regulated by a Dex-induced factor that stimulated P3-IL-1R1 expression in a GR-independent manner.

The differential responses of the macrophage cells and the endothelial cells to the Dex treatment (Fig. 5, B and C) provide an opportunity for us to test whether the differential regulation of IL-1R1 promoters and its consequent cell type-specific modulation of IL-1R1 mRNA expression can translate to cell type-specific responses to IL-1 stimulation. Indeed, Dex treatment is shown to specifically increase IL-1-induced COX-2 expression in RAW 267.7 cells, in which IL-1R1 expression is increased via P1 by Dex, but not in SVEC4–10 cells, in which IL-1R1 expression was unaltered by Dex (Fig. 6). Thus, the promoters discovered in this study may contribute to the diverse response patterns of different cell types to IL-1 stimulation by altering IL-1R1 mRNA expression in a cell type-specific manner.

A detailed comparison between the present study and previous studies that investigated IL-1R1 promoters may serve to further illustrate the significance of present findings. Only the human IL-1R1 promoters were studied in previous studies. Ye et al. (9) were the first to identify a single human IL-1R1 promoter. This promoter, tested by a promoter-reporter assay, was found active in the human monocytic cell line, THP-1. Later, Sims et al. (29) discovered three alternative Exons 1 in human IL-1R1, suggesting three separate promoters initiate human IL-1R1 transcription. This was confirmed in a subsequent study by Ye et al. (10), who revisited their earlier conclusion to assert the existence of three, rather than one, promoters for human IL-1R1. In this study, however, only one of the postulated promoters was experimentally tested in the THP-1 cell line, and 5′ sequences related to all three promoters were reported; their full-length mRNA sequences were not. A major deficiency of these studies is that they did not exclude the possibility that these three human IL-1R1 promoters essentially constitute a single promoter system; only the major promoter drives the production of functional IL-1R1 mRNA, whereas the other two promoters are minor, and they drive the production of IL-1R1 mRNA either in insignificant quantity or in truncated, non-functional form. In contrast, the present study is the first to show that in the mouse genome three different promoters drive the expression of full-length IL-1R1 mRNAs, and all of these mRNAs can be translated to functional IL-1R1 proteins. A significant advance is that the present study demonstrates that there is not a single dominant promoter in murine IL-1R1 gene, and the dominance of individual promoters are controlled in a cell type- and tissue-specific manner. This led us to suspect that the mapping of human IL-1R1 promoters in previous studies might be incomplete, because the previous studies only investigated IL-1R1 mRNA from two different cell lines. We conducted a search for human IL-1R1 promoters using an OriGene human Sure-RACE kit containing cDNA from 24 different tissues to identify human IL-1R1 promoters. This study yielded six different versions of exon 1, suggesting the existence of six promoters for human IL-1R1 (30). No sequence homology was detected between the mouse and human IL-1R1 promoters (data not shown). Further studies are required to elucidate the function of the newly discovered human IL-1R1 promoters, but the fact these discoveries were made only when mRNA from different tissues were analyzed reinforces the key discovery of this study: different promoters drive the expression of IL-1R1 in different tissues.

In summary, the information provided in this study lays an important foundation for further analysis of the intricate regulation of IL-1R1 function. The presence of three promoters for IL-1R1 suggests that different cells may employ different IL-1R1 promoters to activate IL-1R1 transcription. This in turn allows cell type-specific regulation of IL-1R1. For example, P1-dominant cells or tissues may be selectively sensitive to GR-mediated IL-1R1 up-regulation, whereas P2-dominant cells may be selectively regulated when the NF-κB and AP1-mediated signaling pathways are activated (CP2 contains binding sites for NF-κB and AP1). The expression of P3-IL-1R1 in dentate gyrus is interesting because this suggests that IL-1R1 expression in neurons in this region may be controlled differently from other cell types that are involved in IL-1-mediated inflammatory processes. This is consistent with the fact that stimulation of neurons in the brain by IL-1 is not known to produce inflammatory processes but modulates learning and memory by affecting long term potentiation in hippocampal neurons (31). It is of interest to note that a CREB-binding site is only found in CP3. CREB is known to play an essential role in the maintenance of long term potentiation (32). Therefore, controlling IL-1R1 expression by P3 in dentate gyrus may allow the regulation of IL-1R1 expression to be integrated with the role of IL-1 in modulating long term potentiation. As discussed above, whether P3-IL-1R1 contains subtle modification of IL-1R1 protein remains to be determined. If so, P3-IL-1R1 could confer unique functions to neuronal IL-1R1.

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