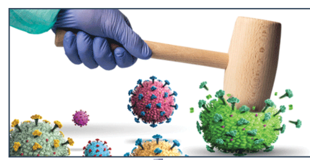
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The 3′ Untranslated Region of the Membrane-Bound IL-1R Accessory Protein mRNA Confers Tissue-Specific Destabilization

Liselotte E. Jensen[1] and Alexander S. Whitehead

IL-1α and IL-1β are proinflammatory cytokines that promote activation of intracellular signaling cascades, leading to stabilization of certain mRNAs and activation of transcription factors. IL-1R type I (IL-1RI) binds IL-1α and IL-1β, and subsequent recruitment of the membrane-bound IL-1R accessory protein (mIL-1RAcP) facilitates signal transduction. Two alternatively spliced isoforms, soluble IL-1RAcP (sIL-1RAcP) and sIL-1RAcP-β, which lack transmembrane and intracellular domains, have been described. The sIL-1RAcP and possibly sIL-1RAcP-β can inhibit IL-1 signaling. Proportional expression of the different IL-1RAcP splice variants may be an important determinant of responsiveness to IL-1. We show that although both mIL-1RAcP and sIL-1RAcP mRNAs are widely expressed in human tissue, their relative proportions differ significantly in a tissue-specific manner. Turnover studies revealed that the sIL-1RAcP mRNA has a half-life of ~48 h in both the kidney cell line 293 and the hepatoma cell line HepG2. The mIL-1RAcP mRNA has a similar half-life in 293 cells, but a considerably shorter half-life of ~5 h in HepG2 cells. Using luciferase reporter constructs, we demonstrated that this specific destabilization of the mIL-1RAcP mRNA in the latter cell type is mediated by its 2.8-kb 3′-untranslated region. Deletion analysis further established that the cell line-specific instability does not involve AU-rich elements, but is mediated by several novel elements that appear to act independently; such elements may be recognized by proteins expressed specifically in some, but not all, tissues. These data demonstrate that the cellular capacity to respond to IL-1 is tightly regulated in a tissue-specific manner. *The Journal of Immunology,* **2004, 173: 6248–6258.**

The IL-1 molecules IL-1α and IL-1β are potent cytokines important for the initiation of systemic inflammatory responses to infections, tissue damage, and stress. Although inflammation is protective in the short term, its prolongation in conditions such as rheumatoid arthritis and Crohn's disease is destructive (reviewed in Ref. 1). In addition, IL-1 may play a role in supporting tumor invasion and angiogenesis (2). Recently, six novel proteins, named IL-1F5 through IL-1F10, with sequence similarity to IL-1 have been identified (3). Although the physiological roles of these novel IL-1 family members are currently ill-defined, recent reports suggest that several have functions similar to those of IL-1 (4, 5).

Cellular responses to IL-1 depend on its binding to the transmembrane receptor IL-1R type I (IL-1RI)[2] and membrane-bound IL-1R accessory protein (mIL-1RAcP) (6–9). The receptor and accessory protein each have three extracellular Ig domains, a short transmembrane region and intracellular regions of 213 and 181 amino acid residues, respectively. Crystallographic studies have revealed that IL-1RI interacts with IL-1 via all three Ig domains (10). Computer modeling has subsequently shown that mIL-1RAcP probably wraps around the IL-1RI · IL-1 complex, such that mIL-1RAcP predominantly interacts with IL-1RI (11). Upon engagement of the receptor complex by IL-1, several adaptor proteins and kinases are recruited to the intracellular domains of both IL-1RI and mIL-1RAcP (reviewed in Ref. 12). Downstream signaling cascades, including the MAPK pathway, are subsequently engaged and lead to a range of cellular responses, including modulation of the stability of a subset of mRNAs (e.g., the TNF, cyclooxygenase-2, and inducible NO synthase mRNAs) (13–15) and activation of transcription factors such as NF-κB and AP-1 (reviewed in Ref. 12). The transcription factors, in turn, up-regulate many genes involved in host defense. It has recently been demonstrated that the novel IL-1-related proteins, IL-1F6, IL-1F8, and IL-1F9, mediate activation of NF-κB and the MAPK signaling cascade via IL-1R-related protein 2 (IL-1Rrp2) and mIL-1RAcP (4, 5).

Several mechanisms have evolved to specifically regulate the activity of IL-1. A classical antagonist, IL-1RA, binds to the first two Ig domains of IL-1RI without initiating signal transduction (16), thereby blocking IL-1 binding and precluding proinflammatory signaling. Clinical trials have demonstrated that administration of IL-1RA can delay joint damage and improve symptoms associated with rheumatoid arthritis (17). The recombinant form of IL-1RA, Anakinra, is currently approved for the treatment of rheumatoid arthritis and is likely to be used for additional therapeutic purposes in the future. Furthermore, IL-1RA has been shown to inhibit tumor growth and angiogenesis in mice (18). Preliminary data suggest that IL-1F5 acts as a potent antagonist of IL-1F9 in a manner similar to that of IL-1RA (5). IL-1R type II (IL-1RII) is a decoy receptor that has an intracellular domain significantly shorter than that of IL-1RI and is therefore unable to recruit the

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[2] Abbreviations used in this paper: IL-1RI, IL-1R type I; CPE, cytoplasmic polyadenylation element; IL-1F, IL-1 family member; IL-1RII, IL-1R type II; IL-1RA, IL-1R antagonist; IL-1RAcP, IL-1R accessory protein; IL-1Rrp2, IL-1R-related protein 2; mIL-1RAcP, membrane-bound IL-1R accessory protein; PRT-PCR, proportional RT-PCR; QRT-PCR, quantitative RT-PCR; sIL-1RAcP, soluble IL-1R accessory protein; UTR, untranslated region.

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adaptor proteins and kinases needed to initiate intracellular signaling (19). An extracellular soluble form of IL-1RII (sIL-1RII) is derived from the membrane-bound form (mIL-1RII) via proteolysis (20–22). Both the membrane-bound and soluble forms of IL-1RII act as ligand sinks for IL-1. However, IL-1RII has low affinity for IL-1RA (23), thereby allowing the antagonist and the receptor decoys to act in concert. In addition to acting as a ligand sink IL-1RII can inhibit IL-1 signaling by forming complexes with IL-1RAcP, which diverts the latter away from the biologically active IL-1RI (24, 25).

As well as mIL-1RAcP, two extracellular soluble forms, sIL-1RAcP and sIL-1RAcP-β, have been identified (7, 26, 27). Unlike sIL-1RII, which is derived by proteolysis of the membrane-bound protein, the soluble forms of IL-1RAcP arise from alternative splicing of transcripts specified by a single gene comprising 12 exons (Fig. 1) (26, 27). Splicing of exon 10 to an alternative splice site within exon 9 yield transcripts encoding mIL-1RAcP, whereas sIL-1RAcP transcripts contain only exons 1–9 (Fig. 1) (26). Elimination of exon 9 from the primary transcript generates mRNA encoding sIL-1RAcP-β (Fig. 1) (27). Both sIL-1RAcP proteins have three full-length extracellular Ig domains, but lack the transmembrane and intracellular domains present in mIL-1RAcP. Soluble IL-1RAcP shares 350 aa with mIL-1RAcP, but has six unique amino acids at the C terminus (26). In contrast, sIL-1RAcP-β shares only 301 aa with mIL-1RAcP (and sIL-1RAcP) and has a distinct 45-aa sequence at the C terminus that comprises a unique second half of the third Ig domain (27). The sIL-1RAcP is present at high levels in human serum (range, 90–600 ng/ml) and enhances the ability of IL-1RII to inhibit IL-1 signaling by increasing its affinity for IL-1 ~100-fold (23). Furthermore, sIL-1RAcP may inhibit IL-1 signaling by directly associating with IL-1RI·IL-1 complexes to competitively prevent the assembly of functional signaling complexes that contain mIL-1RAcP (26). In vivo, sIL-1RAcP has been demonstrated to inhibit collagen-induced arthritis (28). Soluble IL-1RAcP-β may have similar functions. However, the unique C terminus may confer the capacity to interact preferentially with IL-1RI, IL-1RII, or IL-1Rrp2 and a subset of the ligands, IL-1, IL-1F6, IL-1F8, and IL-1F9.

Given the reciprocal roles of mIL-1RAcP and sIL-1RAcP in, respectively, promoting and limiting IL-1 signaling (and possibly IL-1F6, IL-1F8, and IL-1F9 signaling), the proportional expression of these alternatively spliced isoforms is likely to influence the ability of cells to respond to IL-1 family members. We report in this study that different tissues express different relative levels of mIL-1RAcP to sIL-1RAcP. Furthermore, we demonstrate, at least in part, that these distinct expression patterns may arise due to differential tissue-specific instability of the mIL-1RAcP mRNA, and this instability is mediated by elements in the 3′ untranslated region (UTR).

Materials and Methods

Cell lines and treatments

Cell lines were obtained from American Tissue Type Collection (Manassas, VA). Human hepatoma cells (HepG2) were maintained in DMEM with 25 mM HEPES and glutamax-1 (L-alanyl-L-glutamine) supplemented with 10% (v/v) FCS, 1 mM sodium pyruvate, 0.01 mM nonessential amino acids, and 50 μg/ml gentamicin (Invitrogen Life Technologies, Carlsbad, CA). Human embryonic kidney cells (293) were maintained in DMEM with 25 mM HEPES and glutamax-1 (L-alanyl-L-glutamine) supplemented with 10% (v/v) FCS and 50 μg/ml gentamicin. Cells were grown to ~90% confluence and were treated with medium only or combined with 10 ng/ml IL-1α (National Cancer Institute, Frederick, MD) or 5 μg/ml actinomycin D (Sigma-Aldrich, St. Louis, MO). All experiments were performed at least three times with similar outcomes.

Proportional (PRT-PCR) and quantitative (QRT-PCR) RT-PCR

Human total RNA was obtained from BD Clontech (Palo Alto, CA). Isolation of total RNA from cultures and PRT-PCR were performed as described previously (26) with minor modifications. Reverse transcription of 1 μg of total RNA was performed at 42°C using AMV reverse transcriptase (Promega, Madison, WI) and oligo(dN)$_6$ primer (Amersham Biosciences, Piscataway, NJ) in the presence of anti-RNase RNase inhibitor (Ambion, Austin, TX). In brief, specific sIL-1RAcP and mIL-1RAcP PCR products of 357 and 305 bp, respectively, were amplified using a common forward primer (5′-GATGGATTCTCGCAATGAGG-3′) and splice variant specific reverse primers (sIL-1RAcP, 5′-ACTATGGGTTAGATGCCGTC-3′; mIL-1RAcP, 5′-TGAGAATCACCACTAGCAGG-3′; positions are indicated in Fig. 1). Forty cycles of PCR routinely generated levels of products that were optimal for visualization. We have previously established that the proportions of the two splice variants do not change during PCR amplification (26).

For QRT-PCR, the 18S rRNA was used as an internal reference and was amplified (488-bp product) using QuantumRNA 18S Internal Standard (Ambion) primers and competimers (primers that have been modified at the 3′ end to prevent extension) according to the manufacturer's instructions. Products of 357 and 305 bp were amplified, respectively, from sIL-1RAcP

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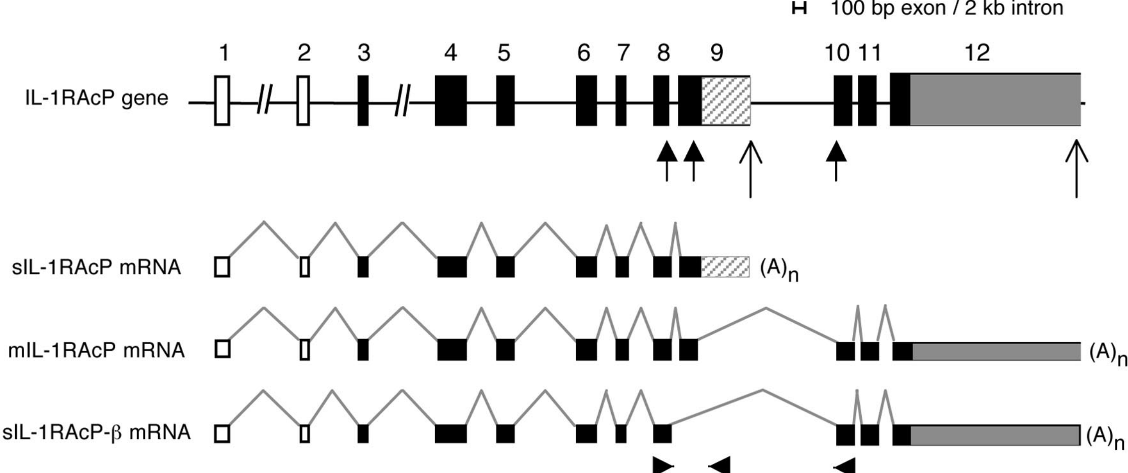

FIGURE 1. Schematic representation of IL-1RAcP gene and alternative splice variants. Exons are shown as wide boxes, introns as thin horizontal lines, and mRNAs as narrow boxes (□, 5′ UTR; ■, coding sequence; ▒, mIL-1RAcP; ▨, sIL-1RAcP 3′ UTR). Open arrows indicate alternative polyadenylation sites, and (A)n represents poly(A) tails. Filled arrows indicate alternative splice sites, and arrowheads at the *bottom* approximate positions of primers used for PCR. The scale bar indicates 100 bp for exon sequence and 2 kb for intronic sequence.

mRNA and mIL-1RAcP mRNA as described above. The use of competimers reduces the efficiency of amplification of rRNA, such that comparable amounts of all three coamplified PCR products are generated after an empirically determined number of PCR cycles. A competimer:primer ratio of 6.5:1 in combination with 23 PCR cycles (determined to be within the exponential phase of amplification) generated optimal and reproducible results.

Amounts of PCR products were quantified after separation in SYBR Green I (Molecular Probes, Eugene, OR) stained with 10% polyacrylamide (29/1, acrylamide/bis-acrylamide) gels using STORM and ImageQuant technology (Molecular Dynamics, Sunnyvale, CA).

Northern blotting

A 262-bp PCR fragment from histone H3 mRNA (positions 178–439 in GenBank accession no. Z49861) were amplified using the primers 5′-CAA CAGTTTCGGATTCATGG-3 and 5′-GTCGGTCTTAAAGTCCTGAG-3′. A histone H3-specific probe was generated from the PCR fragment using oligolabeling (Amersham Biosciences) and [α-^{32}P]dCTP.

Five micrograms of total RNA per sample was separated in 1% denaturing agarose gels and transferred to Hybond-N^{+} filters (Amersham Biosciences). Equal loading of RNA was verified by monitoring levels of ribosomal RNA stained with ethidium bromide. Blots were hybridized sequentially with [α-^{32}P]dCTP-labeled cDNA probes directed against IL-1RAcP and histone H3 mRNA in ULTRAhyb solution (Ambion). Blots were washed extensively, and specific RNA bands were visualized by autoradiography.

Reporter constructs

The full-length 3′ UTRs (polyadenylation signals not included), and fragments thereof, of sIL-1RAcP and mIL-1RAcP mRNAs were amplified by PCR and cloned into the *Xba*I site downstream of the luciferase reporter gene in the pGL3-Promoter vector (Promega). Transcription from this construct is driven by an SV40 promoter. This series of constructs is denominated with the prefix C for constitutive. A series of constructs from which transcription can transiently be induced by IL-1 was also generated and is denominated with the prefix I for inducible. To generate the latter constructs, the E-selectin promoter (−729 to +52 region of the E-selectin gene, positions 112–892 in GenBank accession no. M64485) was cloned into the *Sac*I and *Bgl*II sites upstream of the luciferase gene in the pGL3-Basic vector (Promega). The 3′ UTRs were subsequently subcloned downstream of the luciferase gene in the *Xba*I site. Deletions were made using specific restriction enzymes. Plasmids were treated with two enzymes, and products were separated by electrophoresis. In one series of deletion constructs, the shortened plasmids were religated after blunt-ending of overhanging 5′ and/or 3′ ends. In a second series of deletion constructs, the released insert was blunt-ended and ligated into blunt-ended *Xba*I site in the pGL3-Promoter.

Fragments in which AUUUA-containing regions were deleted were generated through two rounds of PCR amplification. For the first deletion the 5′ end of the *Afl*II-*Bal*I region was amplified using the primers Fdel (5′-GGACCC<u>TCTAGA</u>TAACTTAAGTATTGCTACAG-3′) and del1R (5-AATGATTTATTATGGGTACTGTGTCTGCTGGGAATATAG-3′), and the 3′ end of the *Afl*II-*Bal*I region was amplified using the primers del1F (CTATATTCCCAGCAGACAGAGTACCCATAATAAATCATT-3′) and Rdel (5′-GGACCC<u>TCTAGA</u>CTCTATTGGCCATCTTCTGG-3′). The resulting PCR products were pooled and reamplified using the primers Fdel and Rdel to generate the full-length deletion fragment. For the second deletion the following primers were used: Fdel, Rdel, del2F (5′-TTTGCACTTTTG GATTCCAT . . . GCCTGCCTTTGGTACTTAA-3′), and del2R (5′-TT AAGTACCAAAGGCAGGC . . . ATGGAATCCAAAAGTGCAAAAG-3′). Underlined sequences indicate the positions of *Xba*I sites, and the dotted lines indicate the positions of the deletions.

Fragments of the *Afl*II-*Bal*I region were amplified using the following primers: Fdel and ABF1R (5′-GGACCC*TCTAGA*CATCAACATTCATGT TACCG, (construct C-mIL-1RAcP(*Afl*II-1861); ABF2F (5′-GGACCC*TCTA GA*CGGTAACATGAATGTTGATG-3′) and ABF2R (5′-GGACCC*TCTAG A*TATTCCCTGTTTGCCTAAAG-3′; construct C-mIL-1RAcP(1845–2149); ABF3F (5-GGACCC*TCTAGA*TTTAGGCAAACAGGGAATAG-3′) and Rdel (construct C-mIL-1RAcP(2130-*Bal*I); Fdel and ABF2R, (construct C-mIL-1RAcP(*Afl*II-2149); and ABF2F and Rdel; (construct C-mIL-1RAcP(1845-*Bal*I). The integrity of each construct was confirmed by sequencing.

Transfections and reporter assays

Cells (2.5 × 10^{5}) were grown to ~50% confluence and cotransfected with 50 ng of luciferase reporter construct and 20 ng of *Renilla* luciferase reporter construct (pRL-null; Promega) using 0.5 μl of FuGENE 6 (nonliposomal lipid; Roche, Indianapolis, IN) according to the manufacturer's instructions. In experiments in which cells were transfected with the C series of luciferase reporter constructs, cells were lysed using Passive Lysis buffer (Promega) 24 h after transfection. In experiments in which cells were transfected with the I series of luciferase reporter constructs, cells were treated with IL-1α (10 ng/ml) for 4 h, after which they were rinsed with PBS and fresh medium without IL-1 added (defined as time zero). Cells were lysed at different time points, and luciferase and *Renilla* luciferase activities were determined (Dual-Luciferase Reporter Assay System; Promega) according to the manufacturer's instructions.

Statistical and in silico analyses

Statistical significance of variation in experimental data was tested using a two-sample *t* test.

Homology searches were performed using the Sequence Analysis Tool UTRScan available through the Istituto Tecnologie Biomediche, Consiglio Nazionale delle Ricerche, at web-link http://bighost.area.ba.cnr.it/BIG/UTRHome/, and blastn available through the National Center for Biotechnology Information at http://ncbi.nih.gov/BLAST/.

Sequences of the full-length mIL-1RAcP 3′ UTR and fragments thereof were aligned using ClustalW available through Baylor College of Medicine search launcher at http://searchlauncher.bcm.tmc.edu. Alignments were subsequently analyzed for the presence of conserved RNA folding patterns using the RNA secondary structure prediction server available through Moscow State University, A. N. Belozersky Institute of Physico-Chemical Biology, at http://genebee.msu.su. Individual sequences were folded using the same server and the mfold server available from Rensselaer Polytechnic Institute at http://bioinfo.rpi.edu/applications/mfold.

Results

The sIL-1RAcP and mIL-1RAcP mRNAs are expressed in multiple tissues

To investigate expression of the alternative splice variants of IL-1RAcP mRNA, a panel of tissue total RNA was subjected to PRT-PCR analysis (Fig. 2). Both the sIL-1RAcP and mIL-1RAcP mRNAs were detected in most tissues examined; however, significant differences in their proportional expression were observed. The sIL-1RAcP-β splice variant was not observed in any of the tissue samples analyzed. Colon was the only tissue in which sIL-1RAcP mRNA was the dominant IL-1RAcP splice variant. In kidney, small intestine, stomach, bone marrow, thymus, spleen, mammary gland, testis, uterus, and prostate, mIL-1RAcP was the dominant splice variant, whereas in liver, approximately equal amounts of the two splice variants were observed. In trachea, only the mIL-1RAcP mRNA was detected. Neither of the two IL-1RAcP mRNAs was detected in heart or skeletal muscle tissue, which may reflect either extremely low levels of these species in the specific

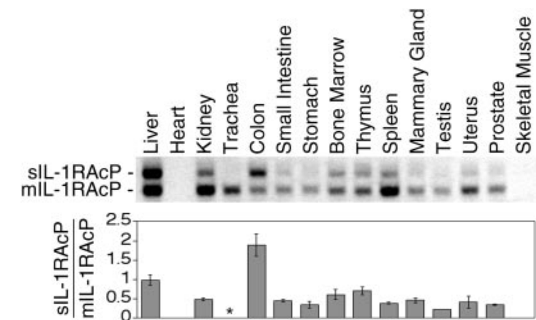

FIGURE 2. Proportional expression of sIL-1RAcP and mIL-1RAcP mRNAs in multiple tissues. Total RNA derived from human tissue was subjected to sIL-1RAcP- and mIL-1RAcP-specific PRT-PCR. PCR products were separated by PAGE (migration pattern shown in *top panel*) and quantitated using STORM imaging, and the ratio of sIL-1RAcP and mIL-1RAcP mRNAs is graphically represented. *, Only mIL-1RAcP mRNA detected; ratio not calculated. Tissue RNA samples were analyzed two or more times with similar outcomes.

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samples and/or stress-induced down-regulation of the mRNAs in these tissues.

The mIL-1RAcP mRNA has a shorter half-life in HepG2 cells than in 293 cells

The alternative splice variants of IL-1RAcP are derived from a single gene (26, 27) (Fig. 1). The simplest mechanism by which two splice variants can be expressed at different relative levels in different tissues is if the stability of one or both mRNAs is differentially governed by tissue-specific factors. To examine some of the potential mechanisms that may underlie tissue-specific differential proportional expression, we initially tested two cell lines that are often used in inflammation research. The cell lines HepG2 (human hepatocellular carcinoma cells with epithelial morphology) and 293 (human embryonic kidney cells with epithelial morphology transformed with adenovirus 5 DNA) are derived from and representative of tissues with apparent differentially regulated proportional expression of the IL-1RAcP splice variants. These cell lines were found to express proportional levels of sIL-1RAcP and mIL-1RAcP mRNAs (0 h; Fig. 3, *A* and *B*) that are comparable to those observed in the corresponding tissue samples (Fig. 2). HepG2 and 293 cells were treated with actinomycin D to block transcription. RNA was extracted at different time points, and levels of IL-1RAcP mRNAs were determined using QRT-PCR (Figs. 3*A* and 8*B*). The effectiveness of actinomycin D in blocking transcription was verified by monitoring levels of histone H3 mRNA, a very short-lived mRNA species, which was completely degraded within 2 h in both cells lines (Fig. 3*C*). The sIL-1RAcP mRNA had a half-life of ~48 h in both 293 (Fig. 3*A*) and HepG2 (Fig. 3*B*) cells based on the linear regression line (29). In contrast, the mIL-1RAcP mRNA had a similar ~48-h half-life in 293 cells (Fig. 3*A*), but a significantly (*t* test of 9 h data: $t = 8.0$; $p < 0.02$) shorter half-life of ~5 h in HepG2 cells (Fig. 3*B*).

The 3′ UTR of the mIL-1RAcP mRNA is a determinant of tissue-specific instability

Regulation of mRNA stability is often mediated by elements within the 3′ UTR. To facilitate identification of regulatory elements within 3′ UTRs, reporter systems using constitutive promoters have been extensively used. We tested a constitutive luciferase reporter model to determine whether the mIL-1RAcP and sIL-1RAcP 3′ UTRs could affect mRNA stability (measured using luciferase levels as a surrogate assay). The sIL-1RAcP (nt 2220–2935; GenBank accession no. AF167340; Fig. 1, ▨) and mIL-1RAcP (nt 703-3483; GenBank accession no. AF167342; Fig. 1, ▩) 3′ UTRs were cloned downstream of the luciferase gene in the pGL3-Promoter plasmid (Fig. 4*A*). Expression from these constructs was driven by the constitutive SV40 promoter. The luciferase reporter construct 1 pGL3-Promoter, construct 2 C-sIL-1RAcP, and construct 3 C-mIL-1RAcP were individually cotransfected with the *Renilla* luciferase reporter construct into HepG2 and 293 cells. Cells were harvested after 24 h, and luciferase and *Renilla* luciferase activities were determined. The presence of the sIL-1RAcP 3′ UTR had no effect on luciferase activity relative to the control value in either HepG2 or 293 cells (Fig. 4*B*). However, the presence of the mIL-1RAcP 3′ UTR in the C-mIL-1RAcP construct resulted in much lower luciferase activity (~10% of that expressed from either the control pGL3-Promoter ($t = 12.3$; $p < 0.01$) or the C-sIL-1RAcP construct ($t = 11.9$; $p < 0.01$)) in HepG2 cells; no such effect was observed in 293 cells (Fig. 4*B*).

Because IL-1 can mediate mRNA stabilization (13), we next examined whether IL-1 could modulate expression from the SV40 promoter luciferase reporter construct 1 pGL3-Promoter, construct 2 C-sIL-1RAcP, and construct 3 C-mIL-1RAcP. Each was individually cotransfected with the *Renilla* luciferase reporter construct into HepG2 and 293 cells. After 24 h, cells were treated with medium only or medium with IL-1. Cells were harvested after 3, 6, 12, 24, or 48 h, and luciferase and *Renilla* luciferase activities were measured. IL-1 did not modify luciferase expression from any of the constructs in HepG2 cells (Fig. 4*C*) or 293 cells (not shown).

The reduced luciferase expression observed from the construct C-mIL-1RAcP reporter construct in HepG2 cells (Fig. 4*B*) could be the result of luciferase mRNA destabilization mediated by the mIL-1RAcP 3′ UTR; however, it could also be due to effects on transcription and/or translation. To further test whether the mIL-1RAcP 3′ UTR confers mRNA instability, we used a transiently inducible reporter model that allows both monitoring of luciferase mRNA turnover and easy standardization for transfection efficiency using *Renilla* luciferase as a reference. We have found that

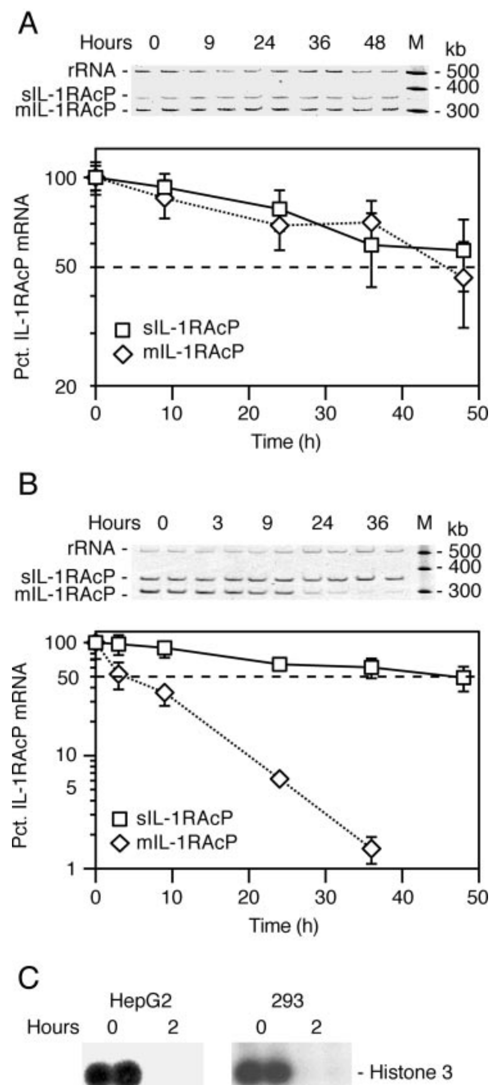

FIGURE 3. The mIL-1RAcP mRNA has a shorter half-life in HepG2 cells than in 293 cells. Cells were treated with actinomycin D, and RNA was harvested at the indicated time points (duplicate samples per time point). Levels of sIL-1RAcP mRNA (solid line) and mIL-1RAcP mRNA (dotted line) in 293 cells (*A*) and HepG2 cells (*B*) were determined using QRT-PCR (*panels*) and are graphically expressed as a percentage of the mRNA level present at the beginning of the experiment. *C*, Histone 3 mRNA levels were determined using Northern blotting. Data shown are representative of three independent experiments.

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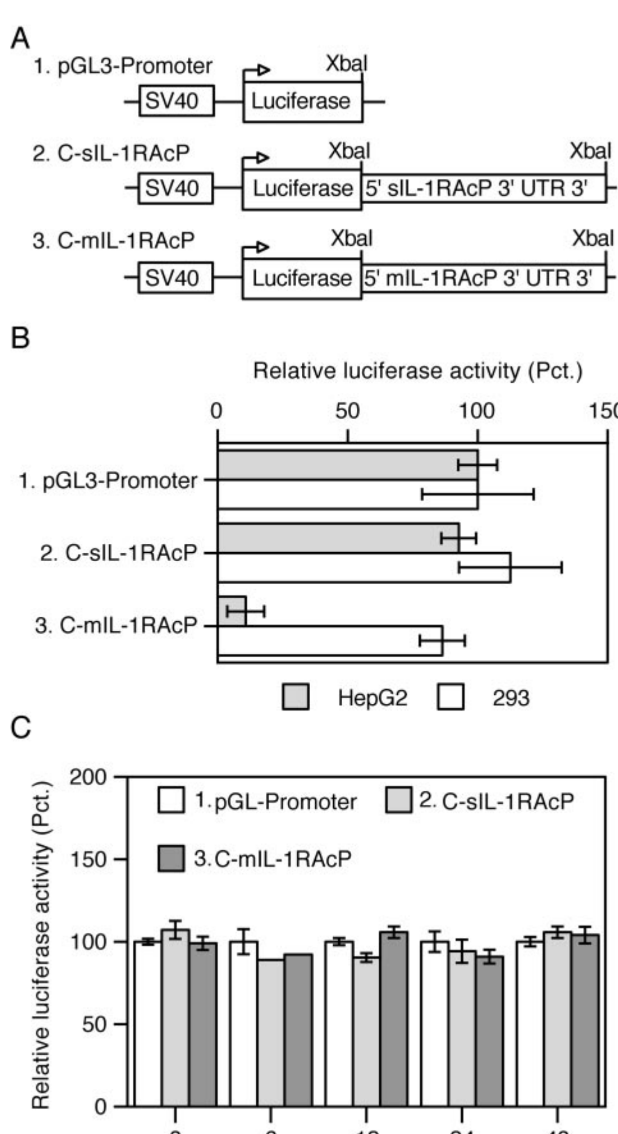

FIGURE 4. Constitutive luciferase reporter constructs reflect instability mediated by the mIL-1RAcP 3′ UTR in HepG2 cells. *A*, Schematic representation of constitutive luciferase reporter constructs (not drawn to scale). Transcription (bent arrow) was driven by the constitutive SV40 promoter upstream of the luciferase gene. The 3′ UTRs of sIL-1RAcP mRNA and mIL-1RAcP mRNA were cloned into the *Xba*I site downstream of the luciferase gene in sense orientation. *B*, The luciferase reporter construct 1 pGL3-Promoter (basic construct without any insertion at the *Xba*I site), construct 2 C-sIL-1RAcP, or construct 3 C-mIL-1RAcP were individually cotransfected with the *Renilla* luciferase reporter construct into HepG2 and 293 cells. Cells were harvested, and the luciferase and *Renilla* luciferase activities were determined. Luciferase activity standardized for transfection efficiency against the *Renilla* luciferase activity (triplicate samples per sampling-point) is graphically represented as a percentage of the activity observed in cells transfected with pGL3-Promoter (activity defined as 100%). *C*, The luciferase reporter construct 1 pGL3-Promoter, construct 2 C-sIL-1RAcP, and construct 3 C-mIL-1RAcP were individually cotransfected with the *Renilla* luciferase reporter construct into HepG2 cells. Cells were treated with medium only or combined with IL-1 (10 ng/ml). Cells were harvested at the indicated time points, and luciferase and *Renilla* luciferase activities were determined. Luciferase activity standardized for transfection efficiency against the *Renilla* luciferase activity (triplicate samples per sampling point) is graphically represented as a percentage of the activity observed in cells transfected with construct 1 pGL3-Promoter (activity defined as 100%). Data shown are representative of three (or more) independent experiments for each construct.

the c-*fos* promoter, which has been used by others as a transiently inducible promoter in reporter assays of mRNA stability (29), is only modestly induced in HepG2 cells (our unpublished observation), and therefore, we chose the E-selectin promoter for use in the above studies. This promoter can be rapidly and transiently activated in response to IL-1 (30), allowing luciferase assays to be used as a surrogate measure of luciferase mRNA degradation after the IL-1 stimulus has been removed, and IL-1-dependent transcription has ceased. We have previously observed that IL-1-dependent E-selectin promoter activity supports maximal luciferase expression after 4 h in HepG2 cells and after 6 h in 293 cells (30) (our unpublished observations). The 3′ UTRs of sIL-1RAcP (Fig. 1, ▨) and mIL-1RAcP (Fig. 1, ▤) were cloned in sense orientation into the E-selectin-luciferase construct at the *Xba*I site downstream of the luciferase-coding sequence (Fig. 5*A*). The resulting luciferase reporter constructs, i.e., a I-pGL3, b I-sIL-1RAcP, and c I-mIL-1RAcP, were individually cotransfected with the *Renilla* luciferase reporter construct into HepG2 and 293 cells before treatment with IL-1 for 4 and 6 h, respectively. Cells were washed with PBS, and fresh medium without IL-1 was added (defined as time zero). In this model transcription has ceased by time zero, i.e., no more luciferase mRNA can be generated. Cells were harvested after 0, 2, 4, 6, 8, 20, 26, and 32 h, and luciferase and *Renilla* luciferase activities were determined. Luciferase activity was standardized (against *Renilla* luciferase) for transfection efficiency and was calculated as the percentage of enzyme activity remaining compared with that present at time zero (for the same reporter construct). The latter ensures that values are not confounded by changes in transcription and/or translation. The above data (Fig. 4*C*) demonstrate that IL-1 does not affect luciferase expression. Therefore, the rate of decline in luciferase activity is a proportional reflection of the rate of luciferase mRNA degradation. In 293 cells, luciferase activity originating from all these constructs declined at similar rates (~50% remaining after 12 h; Fig. 5*B*), indicating that all three reporter transcripts have similar half-lives (t test comparing 8 h data points for construct a I-pGL3 to construct c I-mIL-1RAcP ($t = 0.30$; $p < 0.8$). In HepG2 cells, the apparent half-lives of luciferase mRNA and luciferase-sIL-1RAcP 3′ UTR mRNA were similar to each other (~5 h; Fig. 5*C*) and significantly shorter than in 293 cells, suggesting more rapid turnover of the luciferase mRNA in HepG2 cells. This more rapid turnover appears to be specific to the luciferase mRNA, because the endogenous sIL-1RAcP mRNA has the same half-life in both HepG2 and 293 cells (Fig. 3, *A* and *B*), and may be mediated by elements within the luciferase 5′ UTR and/or coding sequence. Study of the IL-1RAcP 3′ UTRs will not be affected by this HepG2-specific shorter half-life of luciferase mRNA, because expression from the IL-1RAcP 3′ UTR-containing constructs was compared with that from the unmodified luciferase reporter construct in the same cells line. The above data confirm that the sIL-1RAcP 3′ UTR does not influence mRNA degradation. In contrast, the mIL-1RAcP 3′ UTR mandates a dramatically shorter half-life of ~1 h (Fig. 5*C*; t test comparing 8 h data points for a I-pGL3 to c I-mIL-1RAcP: $t = 8.6$; $p < 0.02$), demonstrating that it contains elements that confer mRNA instability in a particular subset of tissues. The data depicted in Fig. 5 do not exclude the possibility that the IL-1RAcP 3′ UTRs affect translational efficiency.

A sequence element(s) located within an 816-bp region is responsible for the mIL-1RAcP tissue-specific destabilization

The full-length mIL-1RAcP 3′ UTR is 2.8 kb (Fig. 6). To determine whether the entire region is required to mandate the differential stability outlined above or whether partial fragments within the 3′ UTR can independently promote a relatively high rate of

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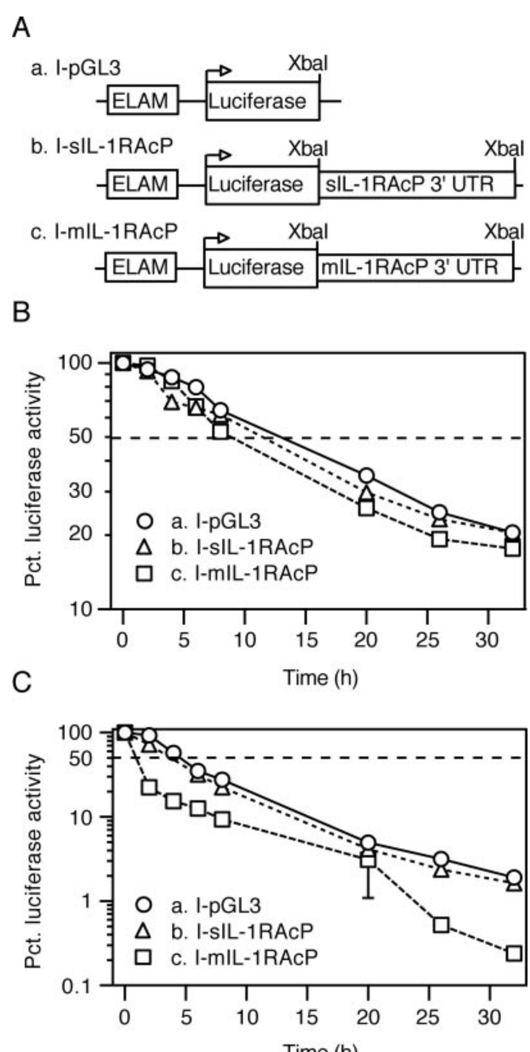

FIGURE 5. The 3′ UTR of the mIL-1RAcP mRNA promotes instability in HepG2 cells. *A*, Schematic representation of IL-1-inducible luciferase reporter constructs (not drawn to scale). Transient transcription (bent arrow) was driven by the IL-1-responsive E-selectin promoter (ELAM) cloned upstream of the luciferase gene. The 3′ UTRs of sIL-1RAcP mRNA and mIL-1RAcP mRNA were cloned into the *Xba*I site downstream of the luciferase gene (construct b I-sIL-1RAcP and construct c I-mIL-1RAcP, respectively). The reporter construct b I-sIL-1RAcP, construct c I-mIL-1RAcP, and construct a I-pGL3 (basic construct without any insertion at the *Xba*I site) were individually cotransfected with the *Renilla* luciferase reporter construct into 293 (*B*) and HepG2 (*C*) cells. Cells were treated with IL-1 for 6 h (293) or 4 h (HepG2), after which they were washed with PBS, and fresh medium without IL-1 was added (defined as time zero). Cells were harvested at different time points (duplicate samples per time point), and luciferase and *Renilla* luciferase activities were determined. Luciferase activities standardized for transfection efficiency against the *Renilla* luciferase activities are graphically represented as a percentage of the activity remaining compared with levels present at time zero using the formula ($[\text{luciferase}_{\text{timeX}}]/[\textit{Renilla}\ \text{luciferase}_{\text{timeX}}])/([\text{luciferase}_{\text{time0}}]/[\textit{Renilla}\ \text{luciferase}_{\text{time0}}]$). The zero hour point is defined as 100%. Data shown are representative of three (or more) independent experiments for each construct.

mRNA degradation, various regions within the mIL-1RAcP 3′ UTR were deleted from the C-mIL-1RAcP luciferase reporter construct using a number of endogenous restriction enzyme sites (Fig. 6). TheC-mIL-1RAcP luciferase reporter construct was digested with two restriction enzymes (to remove a small fragment), blunt-ended, and subsequently religated to generate the deletions series depicted in Fig. 7. Analysis of luciferase activity from construct 1 pGL3-Promoter, construct 3 C-mIL-1RAcP, construct 4 C-mIL-1RAcP(Δ*Xba*I-*Nde*I), construct 5 C-mIL-1RAcP(Δ*Eco*RV-*Spe*I), and construct 6 C-mIL-1RAcP(Δ*Spe*I-*Afl*II) in HepG2 cells revealed that deletion of the *Xba*I-*Nde*I, *Eco*RI-*Spe*I, and *Spe*I-*Afl*II regions did not significantly affect the level of luciferase activity (Fig. 7). In contrast, deletion of the *Afl*II-*Bal*I region (construct 7 C-mIL-1RAcP(Δ*Afl*II-*Bal*I)) resulted in dramatically increased (~6-fold; $t = 35$; $p < 0.001$) luciferase activity compared with that expressed from construct 3 C-mIL-1RAcP (Fig. 7). This suggests that the *Afl*II-*Bal*I region contains sequence elements that are essential for the mIL-1RAcP 3′ UTR-mediated, tissue-specific mRNA instability.

To further characterize the minimal region/element that mandates enhanced degradation of the construct 3 C-mIL-1RAcP transcript (and consequently low luciferase levels), a second series of constructs was generated. Fragments of the mIL-1RAcP 3′ UTR were generated from construct 3 C-mIL-1RAcP by dual restriction enzyme digestion, blunt-ended, and ligated into the blunt-ended *Xba*I site in construct 1 pGL3-Promoter (Fig. 7). An additional construct was generated by removing the region *Spe*I-*Afl*II from construct 8 C-mIL-1RAcP(*Nde*I-*Bal*I). The resulting constructs and C-mIL-1RAcP and pGL3-Promoter constructs were individually cotransfected with *Renilla* luciferase reporter construct into HepG2 cells, and luciferase activities were determined after 24 h. Luciferase activity from construct 8 C-mIL-1RAcP(*Nde*I-*Bal*I) was similar to that from construct 3 C-mIL-1RAcP (Fig. 7). Omission of the regions *Nde*I-*Spe*I (construct 9 C-mIL-1RAcP(*Spe*I-*Bal*I)) or *Spe*I-*Afl*II (construct 10 C-mIL-1RAcP(*Nde*I-*Spe*I+*Afl*II-*Bal*I)) did not affect luciferase activities, suggesting that these regions do not contribute to the relative instability intrinsic to the intact mIL-1RAcP 3′ UTR (Fig. 7). Taken together, the data outlined suggest that the *Afl*II-*Bal*I region contains the element(s) conferring destabilization. The low level of luciferase (~15% that observed from pGL3-Promoter; Fig. 7; $t = 36$, $p < 0.001$ and $t = 33$, $p < 0.001$, respectively) expressed from both construct 1 C-mIL-1RAcP(*Afl*II-*Xba*I) and construct 12 C-mIL-1RAcP(*Afl*II-*Bal*I) reinforces this interpretation and supports the conclusion that the *Afl*II-*Bal*I element is sufficient to confer relative mRNA instability.

Instability specified by the mIL-1RAcP 3′ UTR is not mediated via AUUUA elements

Pentameric AUUUA sequence elements within mRNA 3′ UTRs often confer instability (13, 31). Five such elements are present within the *Afl*II-*Bal*I region of the mIL-1RAcP 3′ UTR (Fig. 6) and are candidates for involvement in controlling tissue-specific instability. Three of these AUUUA sequence elements were eliminated by deleting the 1708–1893 region (numbers in Fig. 6) from the full-length mIL-1RAcP 3′ UTR (Fig. 8*A*; new luciferase reporter construct 13 C-mIL-1RAcP(Δ1708–1893)) or from the *Afl*II-*Bal*I fragment (Fig. 8*B*; construct 15 C-mIL-1RAcP(*Afl*II-*Bal*IΔ1708–1893)). The other two AUUUA sequence elements were removed by deleting the 2212–2273 region both from the full-length construct mIL-1RAcP 3′ UTR (Fig. 8*A*; construct 14 C-mIL-1RAcP(Δ2212–2273)) and from the *Afl*II-*Bal*I fragment (Fig. 8*B*; construct 16 C-mIL-1RAcP(*Afl*II-*Bal*IΔ2212–2273)). The construct 3 C-mIL-1RAcP, construct 13 C-mIL-1RAcP(Δ1708–1893), construct 14 C-mIL-1RAcP(Δ2212–2273), construct 12 C-mIL-1RAcP(*Afl*II-*Bal*I), construct 15 C-mIL-1RAcP(*Afl*II-*Bal*IΔ1708–1893), and construct 16 C-mIL-1RAcP(*Afl*II-*Bal*IΔ2212–2273) luciferase reporters were individually cotransfected with the *Renilla* luciferase reporter construct into HepG2

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FIGURE 6. Sequence of the mIL-1RAcP 3′ UTR. The mIL-1RAcP 3′ UTR is derived from GenBank accession no. AF167342 (positions 702-3508). The mIL-1RAcP mRNA polyadenylation signal and site are located at positions 2783–2788 and 2808, respectively. The luciferase reporter construct c I-pGL3-mIL-1RAcP and construct 3 C-mIL-1RAcP contain the sequence 1–2782. Positions of restriction enzyme sites are indicated in italics and are identified above the sequence. Positions of AUUUA sequence elements are indicated with bold underlining. AUUUA deletion 1 (positions 1708–1893) and AUUUA deletion 2 (positions 2212–2273) are shown with dotted underlining. The positions of CPE and K boxes are shown with thin and double underlining, respectively.

cells. Subsequent analysis of luciferase and *Renilla* luciferase activities revealed that deletion of these regions did not affect expression of the luciferase reporter protein (Fig. 8), suggesting that they are unlikely to be involved in the regulation of tissue-specific expression.

Segments within the Afl*II-*Bal*I region cooperate to promote mRNA instability*

Three subfragments covering the 5′ end (*Afl*II-1861), middle (1845–2144), and 3′ end (2130-*Bal*I) of the *Afl*II-*Bal*I region were generated by PCR and cloned into the *Xba*I site in construct 1 pGL3-Promoter (Fig. 9). The resulting luciferase reporter constructs 17 C-mIL-1RAcP(*Afl*II-1861), 18 C-mIL-1RAcP(1845–2149), and 19 C-mIL-1RAcP(2130-*Bal*I), construct 1 pGL3-Promoter, and construct 12 C-mIL-1RAcP(*Afl*II-*Bal*I) were individually cotransfected with the *Renilla* luciferase reporter construct into HepG2 cells, and luciferase activities were analyzed after 24 h. Luciferase expression from the three constructs containing fragments of the *Afl*II-*Bal*I region, i.e., constructs 17 C-mIL-1RAcP(*Afl*II-1861), 18 C-mIL-1RAcP(1845–2149), and 19 C-mIL-1RAcP(2130-*Bal*I), was significantly higher (2-fold (t = 22.0; $p < 0.001$), 3-fold (t = 16.3; $p < 0.001$), and 3-fold (t = 48.6; $p < 0.001$), respectively) than that from construct 12 C-mIL-1RAcP(*Afl*II-*Bal*I) that contains the entire *Afl*II-*Bal*I region (Fig. 9). However, expression from these three constructs was dramatically lower than that from the pGL3-Promoter reporter constructs (Fig. 9).

We examined the effect of joining two fragments of the three subfragments together (to yield constructs 20 C-mIL-1RAcP(*Afl*II-2149) and 21 C-mIL-1RAcP(1845-*Bal*I)). Decreased luciferase expression was observed from these two fragment-containing constructs compared with those observed from the constructs 17–19 containing only one fragment (Fig. 9). Nevertheless, luciferase expression remained above that observed for the full-length *Afl*II-*Bal*I region (construct 12; Fig. 9; t = 10.7, $p < 0.001$ and t = 8.7, $p < 0.001$, respectively).

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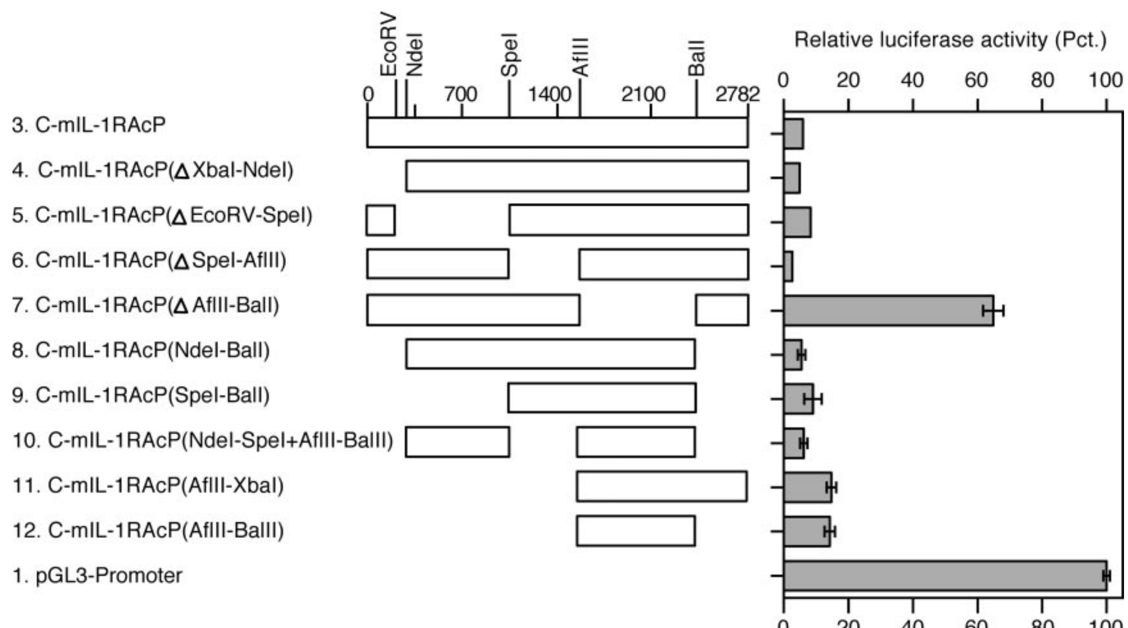

FIGURE 7. Sequence element(s) located between the *Afl*II and *Bal*I restriction sites confers instability in HepG2 cells. Deletions of the 3′ UTR in construct 3 C-mIL-1RAcP were generated using restriction enzyme sites within the full-length mIL-1RAcP 3′ UTR. Deletion luciferase reporter constructs were individually cotransfected with the *Renilla* luciferase reporter construct into HepG2 cells. Cells were harvested 24 h after transfection, and luciferase and *Renilla*-luciferase activities were determined. Luciferase activity standardized against *Renilla* luciferase activity is graphically represented as a percentage of the activity observed in cells transfected with construct 1 pGL3-Promoter (activity defined as 100%). Boxes represent sequence remaining in the respective constructs. Data shown are representative of three (or more) independent experiments for each construct.

The above data suggest that each of the three subfragments contains one or more sequence elements that can independently promote mRNA instability and that they cooperate with each other to achieve the degree of destabilization observed for the full-length mIL-1RAcP 3′ UTR.

The Afl*II*-Bal*I region represents novel 3′ UTR regulatory elements*

The full-length mIL-1RAcP 3′ UTR (sequence 1–2782; Fig. 6) was searched for known 5′ and 3′ UTR regulatory elements using UTRScan. Two K box elements were identified at positions 2330–2335 (within the 3′ end of the *Afl*II-*Bal*I region) and positions 2532–2537, respectively (Fig. 6). An internal ribosome entry site element was identified in the 3′ end of the 3′ UTR (Fig. 6). However, when the sequence 1–2808 (Fig. 6), including the polyadenylation site, was searched, the internal ribosome entry site was not identified, and a cytoplasmic polyadenylation element (CPE) was identified. AU-rich elements were not identified, and no known elements were identified in the 5′ and middle regions of the *Afl*II-*Bal*I region. The *Afl*II-*Bal*I sequence was further searched against the National Center for Biotechnology Information databases of DNA and EST. No significant similarities were identified.

The mfold and RNA secondary structure prediction servers were used to analyze secondary structure(s) of the full-length mIL-1RAcP 3′ UTR and fragments thereof. The two software packages predicted significantly different folding patterns, precluding any conclusions regarding secondary structure.

Discussion

Membrane-bound IL-1RAcP is essential for signaling by IL-1, IL-1F6, IL-1F8, and IL-1F9 (4, 5, 7–9). In contrast, sIL-1RAcP inhibits signaling by IL-1 (23, 26, 28) and possibly IL-1F6, IL-1F8, and IL-1F9. Consequently, regulation of the proportional expression of the IL-1RAcP splice variants is likely to play an important role in modulation of physiological responses to IL-1 and possibly additional IL-1 family members. We have observed that proportions of IL-1RAcP mRNAs vary in a tissue- and/or cell line-specific manner. Tissue-specific proportional expression of the IL-1RAcP isoforms could make certain organs more responsive to IL-1 signaling, but also more susceptible to damage under conditions of chronic inflammation, e.g., induction of apoptosis in pancreatic β-cells, which may lead to type I diabetes (14).

Differential expression of alternative splice variants may involve specific selection of splice and/or polyadenylation sites within the common primary transcript. High rates of transcription may influence the proportions of alternative splice variants if one or more of the proteins involved in processing the primary transcript are present in limiting amounts. Distinct and different stabilities of alternative splice variants can also lead to changes in proportional expression. Such mechanisms may be enhanced if transcription rates differ. We have demonstrated for the first time that levels of mIL-1RAcP mRNA are differentially regulated in different cell lines at least in part via modulation of mRNA stability. The mIL-1RAcP mRNA is specifically destabilized in HepG2 cells, but not in 293 cells, via elements present in its 3′ UTR. The sIL-1RAcP-β 3′ UTR is identical with that of the mIL-1RAcP 3′ UTR (27) (Fig. 1) and would therefore be expected to exhibit similar properties with regard to mRNA stability. In contrast, the 3′ UTR in the sIL-1RAcP mRNA does not appear to contain any such instability elements that impact steady state or induced mRNA levels in a subset of tissues/cell lines. It is therefore likely to be passive in the establishment of differential proportional expression of the different IL-1RAcP splice variants in different tissues/cell lines. The analysis of endogenous IL-1RAcP mRNA stability revealed that IL-1RAcP PCR products were generated in similar amounts relative to the control rRNA product at the beginning of the experiment (0 h; Fig. 3, *A* and *B*) in both HepG2 and 293 cells. This observation suggests that the transcription rate of the IL-1RAcP

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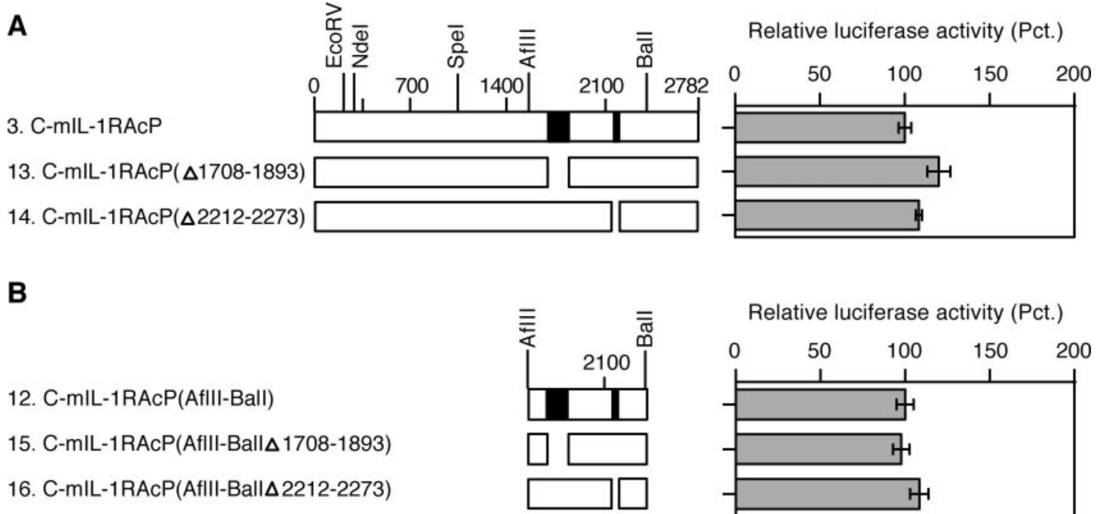

FIGURE 8. AUUUA sequence elements located between the *Afl*II and *Bal*I restriction sites do not confer tissue-specific instability. Two regions containing AUUUA sequence elements were deleted from the 3′ UTRs in construct 3 C-mIL-1RAcP (*A*) and construct 12 C-mIL-1RAcP(*Afl*II-*Bal*I) (*B*) by PCR. Nt 1708–1893 and 2212–2273 (numbers according to Fig. 6) were removed in deletion 1 (Δ1708–1893) and deletion 2 (Δ2212–2273), respectively. Luciferase reporter constructs were individually cotransfected with the *Renilla* luciferase reporter construct into HepG2 cells. Cells were harvested 24 h after transfection, and luciferase and *Renilla* luciferase activities were determined. Luciferase activity standardized against *Renilla* luciferase activity is graphically represented as a percentage of the activity observed in cells transfected with construct 3 C-mIL-1RAcP (*A*) or construct 12 C-mIL-1RAcP(*Afl*II-*Bal*I) (*B*), respectively. □, Sequence remaining in the respective constructs; ■, positions of deletion 1 and deletion 2 within the original construct. Data shown are representative of three (or more) independent experiments for each construct.

gene is similar in the two cell lines and is not a major factor contributing to the differential proportional expression of alternative splice variants.

The 3′ UTRs of mRNAs play important roles in regulating gene expression at the post-transcriptional level. To date, the minimum and maximum lengths of 3′ UTRs observed in human mRNAs are 21 nt and 8.5 kb, respectively, and the average length is 1.0 kb (32). The mIL-1RAcP 3′ UTR, at 2.8 kb, is considerably longer, which suggests that it may have one or more important roles in regulation of gene expression. We have demonstrated that the *Af-lII*-*Bal*I region plays an important role in regulating mIL-1RAcP mRNA levels in HepG2 cells (Fig. 7). Several elements within the *Afl*II-*Bal*I region appear to be involved in mediating mIL-1RAcP mRNA instability and can act independently (Fig. 9). It is possible that a single regulatory protein recognizes and interacts with several elements simultaneously and that the observed decreased luciferase expression from construct 12 C-mIL-1RAcP(AfllII-*Bal*I) compared with any of constructs 17–21 containing fragments of the *Afl*II-*Bal*I region (Fig. 9) is due to increased affinity of such a multibinding protein for the target mRNA 3′ UTR subregion when this is intact. Alternatively, several different proteins may bind to individual 3′ UTR elements and cooperate to achieve full destabilization. Such proteins may be able to bind individually and mediate weaker instability. The precise identification of such specific binding sites is hampered because experimental manipulations to isolate shorter fragments may place them in a primary sequence context that mandates significantly different secondary structures, which may interfere with their recognition by protein factors. We found no significant similarity between the sequence of the mIL-1RAcP 3′ UTR *Afl*II-*Bal*I region and other known 3′ UTRs (see discussion below), suggesting that the *Afl*II-*Bal*I elements represent a novel group of regulatory elements involved in tissue-specific gene regulation. As such, they may interact with one or

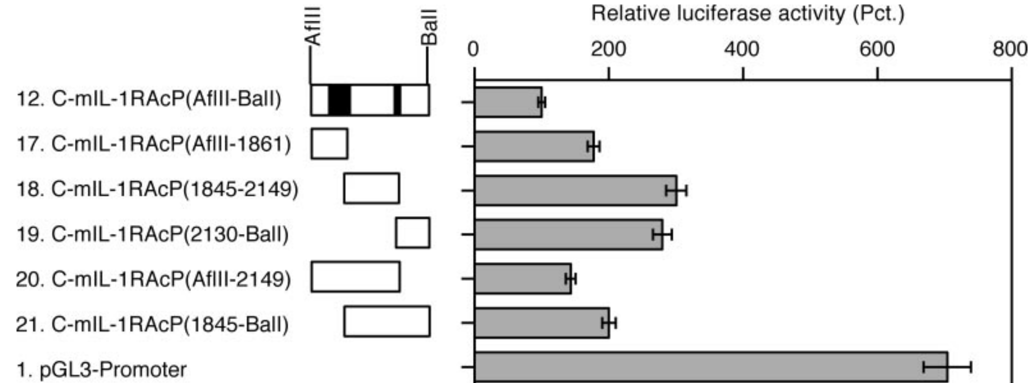

FIGURE 9. Multiple elements within the *Afl*II-*Bal*I region confer instability. Fragments of the *Afl*II-*Bal*I region were generated by PCR and ligated into the *Xba*I site in construct 1 pGL3-Promoter. Luciferase reporter constructs were individually cotransfected with the *Renilla* luciferase reporter construct into HepG2 cells, and luciferase activities were determined after 24 h. Luciferase activity standardized against *Renilla* luciferase activity is graphically represented as a percentage of the activity observed in cells transfected with construct 12 C-mIL-1RAcP(*Afl*II-*Bal*I). □, Sequence remaining in the respective constructs; ■, positions of the AUUUA region deletion 1 and deletion 2 within the original construct. Data shown are representative of three (or more) independent experiments for each construct.

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more proteins that are specifically expressed in liver tissue. Given that construct 12 C-mIL-1RAcP(*Afl*II-*Bal*I) expresses ~2-fold more product than construct 3 C-mIL-1RAcP and construct 7 C-mIL-1RAcP(Δ*Afl*II-*Bal*I) expresses less than construct 1 pGL3-Promoter (Fig. 7), we cannot exclude the possibility that additional elements are present outside the *Afl*II-*Bal*I region. However, we have not been able to determine the position(s) of such a putative site(s). Alternatively, the observed differences in expression levels may be due to alterations in the secondary structure in transcripts from constructs containing shorter 3′ UTR fragments.

Two putative K box elements were identified in the mIL-1RAcP 3′ UTR: one is present in the 3′ end of the *Afl*II-*Bal*I region, and the other is further downstream (Fig. 6). A CPE was also identified in the 3′ end of the full-length mIL-1RAcP 3′ UTR (Fig. 6). K box elements have been demonstrated to be involved in down-regulating mRNA during *Drosophila* development, possibly via interaction with micro-RNAs (33, 34). CPEs regulate translational activation of quiescent maternal mRNAs during late oogenesis and early embryogenesis by promoting cytoplasmic polyadenylation (35). Given that K boxes and CPEs appear to be involved in early development only, and that we observed no effect of deleting the *Bal*I-*Xba*I region, which includes the most downstream K box element and the CPE (Fig. 7), we consider it unlikely that these elements are involved in the cell line-specific destabilization of the mIL-1RAcP mRNA in HepG2 cells reported in this study. However, the presence of these elements is intriguing because they suggest mechanisms by which the expression of mIL-1RAcP might be regulated during early development, e.g., its observed increased expression shortly after trophoblastic elongation (36).

AUUUA pentameric sequences are often involved in regulating mRNA stability and are present in many inflammation-related mRNAs. Activation of the p38 MAPK pathway can lead to stabilization, and hence increased levels, of mRNAs containing AUUUA elements (13). Levels of mIL-1RAcP mRNA have been shown to be modestly up-regulated in liver, spleen, adipose tissue, cerebellum, and the parieto-frontal cortex in response to i.p. LPS (37) and in lung, spleen, and thymus in response to IL-1 (7). The sIL-1RAcP mRNA is up-regulated after intracerebroventricular administration of IL-1 in the hypothalamus and cerebellum (38, 39) and after LPS administration in the hypothalamus (40). The mechanism(s) of these reported changes in IL-1RAcP mRNAs has not been examined, and it is tempting to speculate that it could at least in part be due to induced stabilization of the mRNAs via the AUUUA elements present in both the sIL-1RAcP 3′ UTR and the mIL-1RAcP 3′ UTR. However, we observed no effect on luciferase expression from the reporter constructs containing the sIL-1RAcP 3′ UTR or mIL-1RAcP 3′ UTR after IL-1 treatment (Fig. 4*C*), suggesting that the AUUUA elements are not involved in the regulation of IL-1RAcP mRNA levels. That the AUUUA elements appear to be nonfunctional in this context is further supported by our observation that they do not confer instability in the absence of cytokines (Figs. 7 and 8). Nevertheless, it remains possible that the AUUUA elements are involved in the regulation of IL-1RAcP mRNA stability and levels in tissues and/or cell lines different from those examined in this study.

Regulation of IL-1RAcP expression is clearly very complex, and additional studies will be needed to fully elucidate the mechanisms that govern tissue- and cell line-specific expression of alternative splice variants and stress-induced changes. Identification of the particular factors regulating tissue- and cell line-specific instability of mIL-1RAcP will greatly enhance our understanding of these mechanisms and may facilitate methods by which responsiveness to IL-1 family members can be modulated experimentally and ultimately therapeutically.

References

1. Dinarello, C. A. 1996. Biologic basis for interleukin-1 in disease. *Blood 87:2095*.
2. Voronov, E., D. S. Shouval, Y. Krelin, E. Cagnano, D. Benharroch, Y. Iwakura, C. A. Dinarello, and R. N. Apte. 2003. IL-1 is required for tumor invasiveness and angiogenesis. *Proc. Natl. Acad. Sci. USA 100:2645*.
3. Sims, J. E., M. J. Nicklin, J. F. Bazan, J. L. Barton, S. J. Busfield, J. E. Ford, R. A. Kastelein, S. Kumar, H. Lin, J. J. Mulero, et al. 2001. A new nomenclature for IL-1-family genes. *Trends Immunol. 22:536*.
4. Towne, J. E., K. E. Garka, B. R. Renshaw, G. D. Virca, and J. E. Sims. 2004. Interleukin (IL)-1F6, IL-1F8, and IL-1F9 signal through IL-1Rrp2 and IL-1RAcP to activate the pathway leading to NF-κB and MAPKs. *J. Biol. Chem. 279:13677*.
5. Debets, R., J. C. Timans, B. Homey, S. Zurawski, T. R. Sana, S. Lo, J. Wagner, G. Edwards, T. Clifford, S. Menon, et al. 2001. Two novel IL-1 family members, IL-1δ and IL-1ε, function as an antagonist and agonist of NF-κB activation through the orphan IL-1 receptor-related protein 2. *J. Immunol. 167:1440*.
6. Sims, J. E., M. A. Gayle, J. L. Slack, M. R. Alderson, T. A. Bird, J. G. Giri, F. Colotta, F. Re, A. Mantovani, K. Shanebeck, et al. 1993. Interleukin 1 signaling occurs exclusively via the type I receptor. *Proc. Natl. Acad. Sci. USA 90:6155*.
7. Greenfeder, S. A., P. Nunes, L. Kwee, M. Labow, R. A. Chizzonite, and G. Ju. 1995. Molecular cloning and characterization of a second subunit of the interleukin 1 receptor complex. *J. Biol. Chem. 270:13757*.
8. Wesche, H., C. Korherr, M. Kracht, W. Falk, K. Resch, and M. U. Martin. 1997. The interleukin-1 receptor accessory protein (IL-1RAcP) is essential for IL-1-induced activation of interleukin-1 receptor-associated kinase (IRAK) and stress-activated protein kinases (SAP kinases). *J. Biol. Chem. 272:7727*.
9. Cullinan, E. B., L. Kwee, P. Nunes, D. J. Shuster, G. Ju, K. W. McIntyre, R. A. Chizzonite, and M. A. Labow. 1998. IL-1 receptor accessory protein is an essential component of the IL-1 receptor. *J. Immunol. 161:5614*.
10. Vigers, G. P., L. J. Anderson, P. Caffes, and B. J. Brandhuber. 1997. Crystal structure of the type-I interleukin-1 receptor complexed with interleukin-1β. *Nature 386:190*.
11. Casadio, R., E. Frigimelica, P. Bossu, D. Neumann, M. U. Martin, A. Tagliabue, and D. Boraschi. 2001. Model of interaction of the IL-1 receptor accessory protein IL-1RAcP with the IL-1β/IL-1R(I) complex. *FEBS Lett. 499:65*.
12. Martin, M. U., and H. Wesche. 2002. Summary and comparison of the signaling mechanisms of the Toll/interleukin-1 receptor family. *Biochim. Biophys. Acta 1592:265*.
13. Saklatvala, J., J. Dean, and A. Clark. 2003. Control of the expression of inflammatory response genes. *Biochem. Soc. Symp. 70:95*.
14. Carpenter, L., D. Cordery, and T. J. Biden. 2001. Protein kinase Cδ activation by interleukin-1β stabilizes inducible nitric-oxide synthase mRNA in pancreatic β-cells. *J. Biol. Chem. 276:5368*.
15. Dean, J. L., S. J. Sarsfield, E. Tsounakou, and J. Saklatvala. 2003. p38 Mitogen-activated protein kinase stabilizes mRNAs that contain cyclooxygenase-2 and tumor necrosis factor AU-rich elements by inhibiting deadenylation. *J. Biol. Chem. 278:39470*.
16. Schreuder, H., C. Tardif, S. Trump-Kallmeyer, A. Soffientini, E. Sarubbi, A. Akeson, T. Bowlin, S. Yanofsky, and R. W. Barrett. 1997. A new cytokine-receptor binding mode revealed by the crystal structure of the IL-1 receptor with an antagonist. *Nature 386:194*.
17. Bresnihan, B. 2002. Anakinra as a new therapeutic option in rheumatoid arthritis: clinical results and perspectives. *Clin. Exp. Rheumatol. 20:S32*.
18. Bar, D., R. N. Apte, E. Voronov, C. A. Dinarello, and S. Cohen. 2004. A continuous delivery system of IL-1 receptor antagonist reduces angiogenesis and inhibits tumor development. *FASEB J. 18:161*.
19. McMahan, C. J., J. L. Slack, B. Mosley, D. Cosman, S. D. Lupton, L. L. Brunton, C. E. Grubin, J. M. Wignall, N. A. Jenkins, C. I. Brannan, et al. 1991. A novel IL-1 receptor, cloned from B cells by mammalian expression, is expressed in many cell types. *EMBO J. 10:2821*.
20. Colotta, F., S. Orlando, E. J. Fadlon, S. Sozzani, C. Matteucci, and A. Mantovani. 1995. Chemoattractants induce rapid release of the interleukin 1 type II decoy receptor in human polymorphonuclear cells. *J. Exp. Med. 181:2181*.
21. Orlando, S., M. Sironi, G. Bianchi, A. H. Drummond, D. Boraschi, D. Yabes, and A. Mantovani. 1997. Role of metalloproteases in the release of the IL-1 type II decoy receptor. *J. Biol. Chem. 272:31764*.
22. Orlando, S., C. Matteucci, E. J. Fadlon, W. A. Buurman, M. T. Bardella, F. Colotta, M. Introna, and A. Mantovani. 1997. TNF-α, unlike other pro- and anti-inflammatory cytokines, induces rapid release of the IL-1 type II decoy receptor in human myelomonocytic cells. *J. Immunol. 158:3861*.
23. Smith, D. E., R. Hanna, F. Della, H. Moore, H. Chen, A. M. Farese, T. J. MacVittie, G. D. Virca, and J. E. Sims. 2003. The soluble form of IL-1 receptor accessory protein enhances the ability of soluble type II IL-1 receptor to inhibit IL-1 action. *Immunity 18:87*.
24. Malinowsky, D., J. Lundkvist, S. Laye, and T. Bartfai. 1998. Interleukin-1 receptor accessory protein interacts with the type II interleukin-1 receptor. *FEBS Lett. 429:299*.
25. Lang, D., J. Knop, H. Wesche, U. Raffetseder, R. Kurrle, D. Boraschi, and M. U. Martin. 1998. The type II IL-1 receptor interacts with the IL-1 receptor accessory protein: a novel mechanism of regulation of IL-1 responsiveness. *J. Immunol. 161:6871*.
26. Jensen, L. E., M. Muzio, A. Mantovani, and A. S. Whitehead. 2000. IL-1 signaling cascade in liver cells and the involvement of a soluble form of the IL-1 receptor accessory protein. *J. Immunol. 164:5277*.
27. Jensen, L. E., and A. S. Whitehead. 2003. Expression of alternatively spliced interleukin-1 receptor accessory protein mRNAs is differentially regulated during inflammation and apoptosis. *Cell. Signal. 15:793*.

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28. Smeets, R. L., F. A. van de Loo, L. A. Joosten, O. J. Arntz, M. B. Bennink, W. A. Loesberg, I. P. Dmitriev, D. T. Curiel, M. U. Martin, and W. B. van den Berg. 2003. Effectiveness of the soluble form of the interleukin-1 receptor accessory protein as an inhibitor of interleukin-1 in collagen-induced arthritis. *Arthritis Rheum. 48:2949*.
29. Ross, J. 1995. mRNA stability in mammalian cells. *Microbiol. Rev. 59:423*.
30. Jensen, L. E., and A. S. Whitehead. 2003. ELAM-1/E-selectin promoter contains an inducible AP-1/CREB site and is not NF-κB-specific. *BioTechniques 35:54*.
31. Mignone, F., C. Gissi, S. Liuni, and G. Pesole. 2002. Untranslated regions of mRNAs. *Genome Biol. 3:S0004*.
32. Pesole, G., F. Mignone, C. Gissi, G. Grillo, F. Licciulli, and S. Liuni. 2001. Structural and functional features of eukaryotic mRNA untranslated regions. *Gene 276:73*.
33. Lai, E. C., C. Burks, and J. W. Posakony. 1998. The K box, a conserved 3′ UTR sequence motif, negatively regulates accumulation of enhancer of split complex transcripts. *Development 125:4077*.
34. Lai, E. C. 2002. Micro RNAs are complementary to 3′ UTR sequence motifs that mediate negative post-transcriptional regulation. *Nat. Genet. 30:363*.
35. Stebbins-Boaz, B., and J. D. Richter. 1997. Translational control during early development. *Crit. Rev. Eukaryotic Gene Expression 7:73*.
36. Ross, J. W., J. R. Malayer, J. W. Ritchey, and R. D. Geisert. 2003. Characterization of the interleukin-1β system during porcine trophoblastic elongation and early placental attachment. *Biol. Reprod. 69:1251*.
37. Turrin, N. P., D. Gayle, S. E. Ilyin, M. C. Flynn, W. Langhans, G. J. Schwartz, and C. R. Plata-Salaman. 2001. Pro-inflammatory and anti-inflammatory cytokine mRNA induction in the periphery and brain following intraperitoneal administration of bacterial lipopolysaccharide. *Brain Res. Bull. 54:443*.
38. Plata-Salaman, C. R., and S. E. Ilyin. 1997. Interleukin-1β (IL-1β)-induced modulation of the hypothalamic IL-1β system, tumor necrosis factor-α, and transforming growth factor-β1 mRNAs in obese (*fa/fa*) and lean (*Fa/Fa*) Zucker rats: implications to IL-1β feedback systems and cytokine-cytokine interactions. *J. Neurosci. Res. 49:541*.
39. Gayle, D., S. E. Ilyin, and C. R. Plata-Salaman. 1997. Central nervous system IL-1β system and neuropeptide Y mRNAs during IL-1β-induced anorexia in rats. *Brain Res. Bull. 44:311*.
40. Ilyin, S. E., D. Gayle, M. C. Flynn, and C. R. Plata-Salaman. 1998. Interleukin-1β system (ligand, receptor type I, receptor accessory protein and receptor antagonist), TNF-α, TGF-β1 and neuropeptide Y mRNAs in specific brain regions during bacterial LPS-induced anorexia. *Brain Res. Bull. 45:507*.

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