Characterization of natural antisense transcripts expressed from interleukin 1β-inducible genes in rat hepatocytes

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Abstract

Background: Natural antisense transcripts (asRNAs) are transcribed from many genes in various species. Recently, we found that asRNAs were transcribed from the rat and mouse genes encoding inducible nitric oxide synthase (iNOS), which catalyzes the production of the essential inflammatory mediator nitric oxide. The iNOS asRNA corresponds to the 3'-untranslated region (3'UTR) of the iNOS mRNA. We also found that the iNOS asRNA stabilizes iNOS mRNA through mRNA-asRNA interactions. Single-stranded 'sense' oligonucleotides corresponding to the iNOS mRNA sequence reduce iNOS mRNA levels by blocking mRNA-asRNA interactions; this knockdown method is referred to as natural antisense transcript-targeted regulation (NATRE). It is likely that asRNAs are transcribed from many other inducible genes.

Methods: To detect asRNAs complementary to the 3'UTR of inducible mRNAs, strand-specific reverse transcription-polymerase chain reaction (RT-PCR) was performed. When asRNA was expressed, sense oligonucleotides were designed on the basis of the secondary structure of the mRNA, and NATRE was applied to knockdown the asRNA.

Results: In rat hepatocytes treated with interleukin 1β (IL-1β), we found that asRNAs were transcribed from many inducible genes encoding inflammatory cytokines and chemokines, transcription factors, enzymes, and membrane proteins. When NATRE was applied to these genes, the sense oligonucleotides either down- or up-regulated the mRNA levels. These data support the hypothesis that asRNAs interact with mRNAs to regulate mRNA stability.

Conclusions: We found that asRNAs corresponding to mRNA 3'UTRs were transcribed from many IL-1β-inducible genes. Sense oligonucleotides using NATRE affected the mRNA levels of asRNA-expressing genes, suggesting that the asRNA-mediated regulation of mRNA stability plays a pivotal role. This mechanism may be a general post-transcriptional mechanism that is active during gene induction.

Keywords: Antisense transcript, sense oligonucleotide, knockdown, cytokine, chemokine, NF-κB, TLR
of the iNOS mRNA and stabilizes iNOS mRNA by forming an iNOS mRNA–asRNA–protein complex with the 3′UTR of the mRNA [7]. An AU-rich element (ARE; 5′-AUUUA-3′) in the 3′UTR is involved in mRNA stability [14] and is present in the 3′UTR of the iNOS mRNA and the iNOS asRNA. The ARE-binding protein human homolog R of embryonic-lethal abnormal visual protein (HuR) is assumed to stabilize mRNA in this complex through binding to its 3′UTR [7,15]. In contrast, the ARE-binding protein tristetraprolin (TTP) binds to the ARE and disrupts mRNA stability [16]. There are other examples of asRNAs that regulate gene expression at the post-transcriptional and/or translational levels, such as asRNAs transcribed from the β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) gene and the tumor suppressor gene p53 [17,18].

ARE motifs are frequently found in the 3′UTRs of mRNAs that are transcribed from inducible genes, including early response genes (ERGs), such as cytokines and chemokines [19]. A database analysis revealed that 3′UTRs are the preferred targets of asRNA [20]. Based on these data, it was expected that asRNAs corresponding to the 3′UTR would be transcribed from many genes, particularly ERGs.

We previously found that single-stranded ‘sense’ oligonucleotides corresponding to iNOS mRNA degrade iNOS mRNA [7]. Sense oligonucleotides block interactions between iNOS mRNA and asRNA at single-stranded secondary structure loops and reduce iNOS mRNA stability. Thus, we refer to this knockdown method using sense oligonucleotides as natural antisense transcript-targeted regulation (NATRE; pronounced /nature/) technology [6].

In the present study, using strand-specific RT-PCR, we identified asRNAs transcribed from ERGs that are induced by interleukin 1β (IL-1β) in rat hepatocytes. To examine whether asRNAs interacted with mRNAs, we applied the NATRE technology to the genes that transcribed both mRNAs and asRNAs.

Materials and Methods

Animals: All animal care and experimental procedures were carried out in accordance with the guidelines and laws of the Japanese government and were approved by the Animal Care Committee of Ritsumeikan University, Biwako-Kusatsu Campus. Male Wistar rats (5–6 weeks old) were purchased from Charles River Laboratories, Japan, Inc. (Yokohama, Japan), housed at 21–23°C under a 12-h light-dark cycle with food and water available ad libitum, and acclimatized before the experiment.

The preparation of primary cultured rat hepatocytes. Hepatocytes were isolated from the rat liver by collagenase perfusion, as previously described [21]. Briefly, pentobarbital sodium (65 mg/kg body weight) was intraperitoneally administered, and the liver was perfused with collagenase. Dispersed cells were centrifuged four times. The pellet was resuspended in Williams’ medium E (Sigma-Aldrich Corp., St. Louis, MO, USA) and seeded at 1.2 × 10^6 cells per 35-mm diameter dish for all assays except the transfection assay. The cells were incubated at 37°C for 2 h, and the medium was replaced twice with fresh medium containing 10% newborn bovine serum (SAFC Biosciences, Inc., Lenexa, KS, USA). The purity of the resultant hepatocytes was greater than 99%, as evaluated by microscopic observation (data not shown). The hepatocytes were incubated at 37°C overnight and treated with 1 nM rat IL-1β (PeproTech, Rocky Hill, NJ, USA) for 4 h.

Microarray analysis

Rat hepatocytes were incubated in the presence of IL-1β (1 nM) for 0 or 2.5 h, and total RNA was purified with an RNAqueous kit (Applied Biosystems). Total RNA was subjected to expression analysis using a Rat Genome 230 2.0 GeneChip® Expression Array (Affymetrix Inc., Santa Clara, CA, USA). Expression data were analyzed with a GeneChip Operating Software ver. 1.4 and a Microarray Suite ver. 5.0 (Affymetrix Inc.) as statistical algorithms using Wilcoxon’s signed rank test. To determine ‘increased transcripts’ in the IL-1β-treated hepatocytes (2.5 h) comparing with untreated hepatocytes (0 h), we eliminated probe sets in the experimental sample called “Absent,” selected for probe sets called “Increase,” and eliminated probe sets with a ‘Signal Log Ratio’ of below 1.0.

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was prepared from hepatocytes using the Sepasol I Super (Nacalai Tesque Inc., Kyoto, Japan) and TURBO DNA-free kits (Applied Biosystems, Austin, TX, USA). cDNA was reverse-transcribed in a strand-specific manner with a gene-specific antisense primer or an oligo(dT) primer for mRNA and a gene-specific sense primer for asRNA [7]. Step-down PCR [22] was performed with paired primers, and PCR products were resolved by 2.5% agarose gel electrophoresis and photographed. The primers used in this study are shown in Table 1 and Supplementary Table 1. Alternatively, mRNA levels were estimated in triplicate using real-time PCR with SYBR Green I and the Thermal Cycler Dice Real-Time System (Takara Bio Inc.), as previously described [7]. The obtained values were normalized to elongation factor 1a (EF) mRNA. The sequences of the amplified DNA were confirmed by direct sequencing and deposited in the DNA Data Bank of Japan/European Bioinformatics Institute/GenBank under accession numbers AB302976–AB302978 and AB569422–AB569426.

Determination of the transcript levels. Gene-specific sense primers (Table 1) were used for RT to synthesize cDNAs to the asRNA, and gene-specific antisense primers (Table 1) or an oligo(dT) primer were used to synthesize cDNAs to the mRNA. Real-time PCR was performed with the synthesized cDNA and primer pairs (Table 1) to determine the levels of transcripts. Simultaneously, real-time PCR was performed with known concentrations of gene-specific cDNA fragments as standards. These cDNA fragments were amplified by PCR after RT with a forward primer for an mRNA and a reverse primer for an asRNA. Resultant long cDNA fragments, which harbored the amplified regions for both mRNA and asRNA, were purified by agarose gel electrophoresis and used for real-time PCR as standards of copy numbers. The expression level is shown as number of copies per mg of total RNA used for RT.

Hepatocyte transfection

Hepatocytes (3.0 × 10^6 cells per dish) were transfected in duplicate with a sense oligonucleotide (1.5 μg) using 1.5 μl MATra-A Reagent (IBA, Göttingen, Germany) [7]. The cells were cultured overnight and treated with IL-1β (1 nM) for 4 h before RNA preparation.

Prediction of mRNA secondary structure and the design of sense oligonucleotides. The secondary structures of the mRNA 3′UTR were...
Table 1. Primers used for strand-specific RT-PCR in this study.

| Transcript to be detected | Sequence (5’→3’) | RT/PCR¹ | Direction | cDNA (bp)² |
|---------------------------|------------------|---------|-----------|-----------|
| CCL2 mRNA                 | GGCATCACATTCCAATCACCA | RT      | Reverse   | 228       |
|                           | GCTGCTCAGCCAGATGCACTGTA | PCR     | Forward   |           |
|                           | GATCTCACTGGTTGTCGTCACG | PCR     | Reverse   |           |
| CCL2 asRNA                | ACCAGTATGACAGAAGACTAG | RT      | Forward   | 201       |
|                           | TGTTATTGAAATGATGATGCTTA | PCR     | Forward   |           |
|                           | CAATACCTGGACTCTCACAACAC | PCR     | Reverse   |           |
| CCL20 mRNA                | CATGCACGAAAGACATCGGT | RT      | Reverse   | 223       |
|                           | CAGCCAGTCAGAAAGACGCAACGA | PCR     | Reverse   |           |
|                           | TTGGTTCTTGAGCTGAGGAGGT | PCR     | Reverse   | 201       |
|                           | CAGCCAGTCAGAAAGACGCAACGA | RT      | Forward   |           |
|                           | TAATACCTCCAGATAGGATTACGA | PCR     | Forward   |           |
|                           | TGTTATTGAAATGATGATGCTTA | PCR     | Forward   |           |
|                           | CAATACCTGGACTCTCACAACAC | PCR     | Reverse   |           |
| CCL20 asRNA               | CATGCACGAAAGACATCGGT | RT      | Reverse   | 223       |
|                           | CAGCCAGTCAGAAAGACGCAACGA | PCR     | Reverse   |           |
|                           | TACCTCCAGATAGGATTACGA | PCR     | Forward   |           |
| CD69 mRNA                 | GTGCCCTATTCCATGAGT | RT      | Reverse   | 331       |
|                           | CGCTCGTCAGATAGGATTACGA | PCR     | Forward   |           |
|                           | ATTCATATGACAGAAGACGCAACGA | PCR     | Reverse   |           |
|                           | TACCTCCAGATAGGATTACGA | PCR     | Forward   |           |
|                           | ATTCATATGACAGAAGACGCAACGA | PCR     | Reverse   |           |
| CX3CL1 mRNA               | ACCAGGAGCACGACTGCTGCTGTGCTG | RT      | Reverse   | 149       |
|                           | ATGGTGAATGATGATGATGATGATG | PCR     | Forward   |           |
|                           | GATCTGTCTCAGATGATGATGATG | PCR     | Reverse   |           |
| CX3CL1 asRNA              | ACCAGGAGCACGACTGCTGCTGTGCTG | RT      | Reverse   | 200       |
|                           | ATGGTGAATGATGATGATGATGATG | PCR     | Forward   |           |
|                           | GATCTGTCTCAGATGATGATGATG | PCR     | Reverse   |           |
| EF mRNA                   | TCTGATTCATGAGGATTACGA | RT      | Reverse   | 335       |
|                           | CCAGAGAAGACCTCCACTCAAACGCA | PCR     | Forward   |           |
|                           | GGTGACAGAAGATGATGATGATG | PCR     | Reverse   |           |
| IκB-α mRNA                | GAGGAGGAGACGACTGCTGCTGTGCTG | RT      | Reverse   | 149       |
|                           | ATGGTGAATGATGATGATGATGATG | PCR     | Forward   |           |
|                           | GATCTGTCTCAGATGATGATGATG | PCR     | Reverse   |           |
| IκB-α asRNA               | TCCAGAATCTGTGAAATAGGACCAC | RT      | Reverse   | 107       |
|                           | TAGAAGGCTGCAACTGATGCTGCTG | PCR     | Forward   |           |
|                           | GAAATGGTAGCCTAATCTGCTGCTG | PCR     | Reverse   |           |
| IL-23A mRNA               | CATGGGAACCTGGCAGATCCTT | RT      | Reverse   | 176       |
|                           | CAAGAGAAGACGACTGCTGCTGCTG | RT      | Reverse   | 176       |
|                           | GGTGACAGAAGATGATGATGATG | PCR     | Reverse   |           |
| IL-23A asRNA              | CATGGGAACCTGGCAGATCCTT | RT      | Reverse   | 205       |
|                           | CAAGAGAAGACGACTGCTGCTGCTG | RT      | Reverse   | 205       |
|                           | GGTGACAGAAGATGATGATGATG | PCR     | Reverse   |           |
| NF-κB p65 mRNA            | ACCCTCTCCAAGTCCCTCATAGA | RT      | Reverse   | 262       |
|                           | ACCTTAAATCACATCAGTCTAGCCA | PCR     | Reverse   |           |
|                           | GGTGACAGAAGATGATGATGATG | PCR     | Reverse   |           |
| NF-κB p65 asRNA           | CATGGGAACCTGGCAGATCCTT | RT      | Reverse   | 265       |
|                           | TATGCGTAGCTTCTTCTGACATG | PCR     | Reverse   |           |
|                           | TCCCTCTGATTAAAGGCACTGGA | PCR     | Reverse   |           |
| NF-κB p50 mRNA            | CAGCTCTGCTGGAAGGTCGACGCC | RT      | Reverse   | 254       |
|                           | GTATTGATACATACCTGCTCAGTGC | PCR     | Reverse   |           |
|                           | CTGATGAGCCTGAGGTTGACGCC | PCR     | Reverse   |           |
| NF-κB p50 asRNA           | CTGATGAGCCTGAGGTTGACGCC | RT      | Reverse   | 192       |
|                           | CTGATGAGCCTGAGGTTGACGCC | PCR     | Reverse   |           |

¹ Gene-specific sense primers used for reverse transcription (RT) to synthesize complementary DNA (cDNA) to the antisense transcript (asRNA). A gene-specific antisense primer or an oligo(dT) primer used to synthesize cDNAs for each mRNA. Primer pairs used for polymerase chain reaction (PCR).
² The size of the cDNA fragment amplified by each pair of PCR primers is shown in base pairs (bp).

CCL, chemokine C-C motif ligand; CX3CL1, chemokine C-X3-C motif ligand 1; EF, elongation factor 1α; IκB-α, inhibitor of nuclear factor κB α; IL-23A, interleukin 23, α subunit; and NF-κB, nuclear factor κB.
The results in the figures are representative of at least three independent experiments that yielded similar findings. The values are represented as the mean ± standard deviation (SD). Differences were analyzed with Student’s t-tests. Statistical significance was set at P<0.05 and P<0.01.

Table 2. The expression levels of the mRNA and asRNA in IL-1β-treated hepatocytes

| Gene       | asRNA level [Sense primer] (copies/μg RNA) | mRNA level [Antisense primer] (copies/μg RNA) | mRNA level [AS]: asRNA ratio | mRNA level [Oligo(dT) primer] (copies/μg RNA) | mRNA level [dT]: asRNA ratio |
|------------|------------------------------------------|---------------------------------------------|------------------------------|-----------------------------------------------|-------------------------------|
| IL-23A     | 4.38 × 10⁵                                | 7.71 × 10⁴                                | 1.8                         | 1.01 × 10⁶                                  | 2.3                           |
| CCL2       | 2.30 × 10⁴                                | 1.20 × 10⁴                                | 521.6                       | 1.51 × 10⁷                                  | 655.6                         |
| CCL20      | 8.24 × 10⁴                                | 5.05 × 10⁴                                | 61.3                        | 1.10 × 10⁷                                  | 133.9                         |
| CX3CL1     | 9.70 × 10³                                | 2.10 × 10³                                | 2.2                         | 8.28 × 10⁵                                  | 8.5                           |
| CD69       | 5.37 × 10³                                | 3.87 × 10³                                | 720.2                       | 7.98 × 10⁶                                  | 1484.6                        |

1. A gene-specific sense primer was used for RT to synthesize cDNA to the asRNA. To determine the asRNA levels, real-time PCR was performed with the cDNA using known concentrations of gene-specific cDNA fragments as standards, as described in Materials and Methods. The expression level is shown as number of copies per mg of total RNA used for RT.
2. A gene-specific antisense primer (AS) or an oligo(dT) primer was used in RT to synthesize cDNA to the mRNA, and real-time PCR was performed to determine the copy numbers of the mRNA.
3. The mRNA:asRNA ratio was calculated by dividing the mRNA level by the asRNA level. IL-23A, interleukin 23, α subunit; CCL, chemokine C-C motif ligand; and CX3CL1, chemokine C-X₃-C motif ligand 1.

**Results**

**IL-1β-induced mRNA expression in rat hepatocytes**

It was expected that the asRNAs transcribed from inducible genes (ERGs) would correspond to the 3'UTRs of each mRNA [6,20]. We performed a microarray analysis to compare the mRNA expression profile in rat hepatocytes treated with IL-1β for 2.5 h to untreated hepatocytes (0 h). We eliminated probe sets (i.e., transcripts) in the 2.5-h sample called Absent, and 17,393 probe sets were detected among 31,042 probe sets of a GeneChip at 2.5 h after the addition of IL-1β (data not shown). By selecting for probe sets called Increase, an IL-1β-induced signal increase at 2.5 h was detected for 2,578 transcripts (14.8% of the total expressed transcripts). Signals showing at least a two-fold increase were detected for 592 transcripts (3.4%), which were assumed to be the transcripts that were induced by IL-1β.

The expression of asRNAs corresponds to cytokine and chemokine mRNAs

Next, to detect asRNAs that were complementary to 3'UTRs, strand-specific reverse transcription polymerase chain reaction (RT-PCR) was performed [22]. This sensitive method uses gene-specific sense primers for RT (Table 1) to detect asRNAs that are expressed at relatively low levels [5,6]. The sense primer hybridizes with asRNA and is used to synthesize complementary DNA (cDNA). In contrast, mRNA was detected after RT with a gene-specific antisense primer or an oligo(dT) primer, which hybridizes with the poly(A) tail. Strand-specific RT-PCR was applied to the above-mentioned inducible genes. As shown in Figure 1, we detected bands for asRNAs transcribed from many inducible genes, including the α subunit p19 of IL-23 (IL-23A), chemokine C-C motif ligand (CCL) 2, CCL20, and chemokine C-X₃-C motif ligand 1 (CX3CL1). Sequence analyses of the amplified cDNAs demonstrated that the asRNA corresponded to the mRNA 3'UTR (data not shown). Furthermore, we detected bands for the mRNAs and confirmed their induction by IL-1β (Figure 1). Similar results were obtained, when an oligo(dT) primer was used for RT. These data suggest that these genes transcribed both mRNA and asRNA in response to IL-1β.

We next determined the levels of the asRNA and mRNA by predicted by the mfold program [23]. Regions that were conserved among predicted 3'UTR structures were selected. Each conserved region included at least one stem-loop structure. The sequences of sense oligonucleotides designed according to the mRNA sequence included at least one single-stranded loop in the conserved region, and CpG motifs and G-quartets were avoided [7]. Oligonucleotides blocked by phosphorothioate bonds (GeneDesign Inc., Ibaraki, Osaka, Japan) were used for transfection (Table 4).
Table 3. The expression of asRNAs from IL-1β-inducible genes in rat hepatocytes

| Group | ARE1 | as-RNA | No. of genes | Gene product2 |
|-------|------|--------|--------------|---------------|
| 1     | (+)  | (+)    | 16           | CCL2, CCL20, CD69, CXCL1, CX3CL1, FAM89A, GPRK, IL-23A, INOS, IκB, NF-κB p65, NF-κB p50, phospholipid scramblase 1, RUNX1, semaphorin 4A, STEAP4 |
| 2     | (+)  | (–)    | 5            | Calcitonin-related polypeptide β, carbonic anhydrase II, CCL7, IL-1β, SMPD3 |
| 3     | (–)  | (+)    | 3            | Lymphotoxin β, PSMB10, Toll-like receptor 2 |
| 4     | (–)  | (–)    | 1            | CDKN2B |
| Total |       |        | 25           |               |

1AU-rich element(s) (ARE) present (+) or absent (–) in the mRNA 3’UTR.
2Genes that were significantly induced (i.e., at least a 2-fold increase in signals) in a microarray analysis are shown. Genes to which the NATRE technology was applied are underlined. CXCL1, chemokine C-X-C motif ligand 1; FAM89A, family with sequence similarity 89, member A; GPRK, G protein-coupled receptor kinase 5; INOS, inducible nitric oxide synthase; RUNX1, runt-related transcription factor 1; STEAP4, six-transmembrane epithelial antigen of prostate 4; SMPD3, neutral sphingomyelin phosphodiesterase 3; PSMB10, proteasome subunit, β type 10; CDKN2B, cyclin-dependent kinase inhibitor 2B.

Figure 2. The expression of NF-κB mRNA and asRNA in IL-1β-treated hepatocytes.

Figure 3. Structures of the mRNA 3’UTR and detection of the asRNA. The 3’-termini of various mRNAs are schematically shown. The coding sequence (CDS) and the 3’UTR, both of which correspond to the last exons, are aligned to the stop codons. The size of the 3’UTR without a poly(A) tail (An) is: 642 nucleotides (nt) (IL-23A), 258 nt (CCL2), 454 nt (CCL20), 1855 nt (CX3CL1), 940 nt (CD69), 499 nt (IκB, a), 729 nt (NF-κB p65), and 683 nt (NF-κB p50). Location of AREs in the 3’UTRs are depicted by rhomboids, and numbers of the ARE motifs are indicated in the right side. The AREs conserved between rat and human are shown by green rhomboids. Gene-specific sense primers for RT and the regions amplified by PCR to detect the asRNAs are indicated by blue arrowheads and double-headed arrows beneath the 3’UTRs. The asRNAs corresponded to these amplified regions indicated by double-headed arrows.

real-time PCR using known concentrations of gene-specific cDNA standards and compared the levels (Table 2). The mRNA:asRNA ratios varied from 1.8 to 72, when gene-specific antisense and sense primers (Table 1) were used for strand-specific RT of mRNA and asRNA, respectively. When an oligo(dT) primer was used for RT of mRNA, the mRNA:asRNA ratios showed similar results ranging from 2.3 to 1485. These data demonstrated that the levels of these asRNAs (IL-23A, CCL2, CCL20, CX3CL1, and CD69) are much lower than the levels of corresponding mRNAs.

In addition, asRNAs corresponding to lymphotoxin β and
chemokine C-X-C motif ligand 1 (CXCL1) mRNAs were also detected, whereas asRNAs corresponding to IL-1β and CCL7 mRNAs were not detected (data not shown).

The expression of asRNAs corresponding to mRNAs encoding transcription factors and enzymes.

We next examined the expression of asRNAs corresponding to the 3'UTR of mRNAs encoding the transcription factor nuclear factor κB (NF-κB) in the IL-1β signaling pathway. As shown in Figure 2, asRNAs corresponding to subunits p65 and p50 of NF-κB mRNA were expressed in rat hepatocytes. asRNA corresponding to IκB-α (inhibitor of NF-κB α), which regulates the translocation of NF-κB to the nucleus \( [24] \), was also expressed. Furthermore, an asRNA corresponding to runt-related transcription factor 1 (RUNX1) mRNA was expressed (data not shown).

asRNAs transcribed from genes encoding various enzymes were also detected by strand-specific RT-PCR for G protein-coupled receptor kinase 5 (GPRK5) and phospholipid scramblase 1, although asRNAs corresponding to carbonic anhydrase II and neutral sphingomyelin phosphodiesterase 3 (SMPD3) mRNAs were not detected (data not shown). In addition, asRNA transcribed from genes encoding membrane proteins, such as CD69 (Figure 1), six-transmembrane epithelial antigen of prostate 4 (STEAP4) and Toll-like receptor 2 (TLR2; data not shown), were expressed.

The classification of asRNA expression by the presence of ARE motifs

All of the results of the strand-specific RT-PCR analyses of the IL-1β-inducible genes are summarized in Table 3. All of these genes showed a significant increase in mRNA expression in response to IL-1β. Our data demonstrate that asRNAs were transcribed from 19 inducible genes among the 25 genes analyzed (76% of the total expressed genes). When we classified the 19 asRNA-expressing genes with respect to the presence of ARE motifs in the 3'UTR, we found that 16 genes transcribed mRNA that harbored one or more ARE motifs (84% of the total asRNA-expressing genes). For example, as shown in Figure 3, ARE motifs are present in the 3'UTRs of these 16 genes and are conserved among rat, mouse, and humans (data not shown). These data suggest that asRNAs correlating to the mRNA
3'UTR were frequently transcribed from IL-1β-inducible genes in hepatocytes. This result confirms the hypothesis that asRNAs are widely expressed [6,25].

Interestingly, asRNAs were expressed from the genes encoding lymphotoxin β, proteasome subunit β type 10 (PSMB10), and TLR2, even though their mRNAs do not harbor any ARE motifs in the 3'UTR. These results imply that asRNA expression is not dependent on the presence of an ARE motif in the mRNA 3'UTR.

**Sense oligonucleotides affect mRNA levels**

When asRNA-mRNA interactions are present, single-stranded sense oligonucleotides introduced by NATRE technology may change the mRNA levels [6,7]. Therefore, we applied NATRE technology to the IL-1β-inducible genes from which both mRNA and asRNA were abundantly transcribed. Sense oligonucleotides were designed based on the secondary structures predicted by the mfold program [23] because the loops are assumed to be the sites of the asRNA-mRNA interactions [7]. Each sense oligonucleotide had an identical sequence to the corresponding mRNA and included at least one single-stranded region (loop or bulge) that was conserved among the structures predicted by the mfold program [23] (Table 4, underlined).

Sense oligonucleotides corresponding to each asRNA-expressing gene were introduced into rat hepatocytes and incubated in the presence of IL-1β, and total RNA was prepared from the cells. Real-time PCR analyses of the total RNA indicated that the sense oligonucleotide against IL-23A mRNA significantly decreased the level of IL-23A mRNA (Figure 4), similar to sense oligonucleotides for iNOS [7]. In contrast, sense oligonucleotides for CCL2, CCL20, CX3CL1, CD69, and NF-κB p65 mRNA significantly increased the levels of the corresponding mRNAs. However, most sense oligonucleotides in this study affected the mRNA levels in some way, showing that the knockdown of asRNA by a sense oligonucleotide can not only down-regulate but also up-regulate the corresponding mRNA. These results also support the hypothesis that asRNAs interact with

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**Figure 5. The regulation of mRNA stability by a sense oligonucleotide using NATRE technology.**

Schematic models of the regulation of mRNA stability by a sense oligonucleotide are shown. The sense oligonucleotide acts as an effector to control mRNA levels through an asRNA-mediated post-transcriptional regulation mechanism.

(A) A sense oligonucleotide down-regulates mRNA levels. When mRNA interacts with asRNA and a stabilizing protein, such as HuR, the mRNA is stabilized. When a sense oligonucleotide is introduced, it competes with mRNA to block asRNA-mRNA interactions. This inhibition results in the degradation of the mRNA (broken line) because the stabilizing protein is dissociated from the mRNA. Down-regulation of the mRNA level occurs, such as when sense oligonucleotides corresponding to iNOS [7] and IL-23A mRNA were used.

(B) A sense oligonucleotide up-regulates mRNA levels. The mRNA becomes unstable when a destabilizing protein, such as TTP, binds to the mRNA. The sense oligonucleotide competes with the mRNA to block the asRNA-mRNA interactions, resulting in the dissociation of the destabilizing protein from mRNA. Up-regulation of mRNA levels occurs, such as when sense oligonucleotides corresponding to CCL2, CCL20, CX3CL1, CD69, and NF-κB p65 mRNA were used.
mRNAs to regulate mRNA stability [6,25].

Discussion

Our strand-specific RT-PCR analyses showed that 19 (76%) of 25 IL-1β-inducible genes in hepatocytes transcribed asRNAs, and 16 (84%) of the 19 asRNA-expressing genes harbored ARE motifs in the 3'UTR of the mRNA (Table 3). Many ARE motifs in the 3'UTRs of these 16 mRNAs were conserved among rat, mouse, and humans (Figure 3). We clearly demonstrated that many asRNAs corresponding to the 3'UTR of the mRNA were transcribed from the inducible genes, i.e., ERGs. It is likely that the inducible genes expressing asRNAs tend to have AREs in the mRNA 3'UTR. Our data are in agreement with a report that the 3'UTR is a preferential target of asRNA [20]. The asRNAs were transcribed from a variety of genes encoding cytokines, chemokines, transcription factors, enzymes, and membrane proteins (Table 3). Similar to the asRNAs from the iNOS, BACE1, and p53 genes [7,17,18], the asRNAs detected in this study were expected to regulate the stability of the IL-1β-inducible mRNAs through the 3'UTR.

Real-time PCR showed that the mRNA:asRNA ratios varied from 1.8 to 720, when gene-specific antisense primers were used for RT (Table 2). Because the asRNA tends to be poly(A)-negative [5], and the expression levels of asRNA are relatively low [5,6], we used an oligo(dT) primer for RT of mRNA. When the oligo(dT) primer was used for RT, the mRNA:asRNA ratios were ranging from 2.3 to 1485, i.e., 1.3- to 3.9-fold higher than those when the antisense primers were used. These data demonstrate that these asRNA levels were lower than the levels of corresponding mRNAs. In addition, melting temperature of an oligo(dT) primer (12-18 nt) is much lower than those of the antisense primers (21 nt). Therefore, the oligo(dT) primer may more efficiently hybridize to mRNA and prime cDNA synthesis, resulting in an increase of the mRNA:asRNA ratios.

Indeed, the sense oligonucleotides introduced using NATRE technology successfully regulated the mRNA levels of the asRNA-expressing genes (Figure 4). These results imply that sense oligonucleotides block mRNA-asRNA interactions that normally regulate mRNA stability. Because each sense oligonucleotide includes at least one loop or bulge in the mRNA secondary structure, it may compete with mRNA, thus inhibiting the mRNA-asRNA interaction. Accordingly, when an oligonucleotide hybridizes to an asRNA, it blocks the mRNA-asRNA interactions and causes a conformational change of mRNA and a decrease in the affinity of the stabilizing protein HuR, modulating mRNA stability [26]. In this study, many sense oligonucleotides significantly altered the corresponding mRNA levels, indicating that our secondary structure-based design produced effective sense oligonucleotides. These results suggest that the asRNA-mediated post-transcriptional mechanism is an essential mechanism during gene induction.

The mRNA level was down- or up-regulated by each sense oligonucleotide (Figure 4). The mRNA-asRNA interaction is assumed to be a trigger to recruit RNA-binding proteins, such as ARE-binding proteins [7]. During the recruitment process, there are two possible mechanisms through which mRNA levels can be regulated by asRNAs. Initially, a sense oligonucleotide causes a conformational change in the mRNA. Then, this change leads to the dissociation of an RNA-binding protein that stabilizes mRNA (e.g., HuR), resulting in the degradation of the mRNA (Figure 5A). IL-23A mRNA (this study) and iNOS mRNA [7] are examples of this case. In contrast, an RNA-binding protein that destabilizes the mRNA (e.g., TTP [16]) dissociates from the mRNA, thus stabilizing the mRNA (Figure 5B). The CCL2, CCL20, CX3CL1, CD69, and NF-κB p65 mRNAs fall into this category. Similarly, knockdown of asRNA by sense oligonucleotides increases the levels of brain-derived neurotrophic factor (BDNF) mRNA and glial-derived neurotrophic factor (GDNF) mRNA in vitro [27]. Because structural changes in RNA are the basis of genetic regulation [28], secondary structural transitions may be essential when a sense oligonucleotide is introduced into the cell. Recent studies showed that microRNAs are also involved in mRNA stability, and asRNA inhibits microRNA functions [29]. More studies should be performed to clarify the mechanisms through which asRNA regulates the mRNA level.

Accumulating evidence supports the hypothesis that asRNAs are commonly and widely expressed, and they are assumed to be an essential regulatory RNA in gene expression. In this study, we focused on asRNAs that correspond to the 3'UTR of mRNAs, which is deeply involved in mRNA stability. Blocking asRNA function through sense oligonucleotide-mediated NATRE technology may be a useful method for identifying the sites of mRNA-asRNA interactions and a potential therapy to regulate mRNA levels. Recently, Modarresi et al., reported that the knockdown of BDNF mRNA increases BDNF protein expression in the mouse brain [27]. We are currently examining the therapeutic potential of NATRE technology that targets asRNA-mediated post-transcriptional gene regulation.

Conclusions

We showed that asRNAs corresponding to the 3'UTRs of mRNAs were transcribed from many IL-1β-inducible genes in rat hepatocytes. Sense oligonucleotides introduced using NATRE technology affected the mRNA levels of the asRNA-expressing genes. The present study suggests that the asRNA-mediated regulation of mRNA stability is a general post-transcriptional mechanism that acts during gene induction.

Additional material

Supplementary tables. This file contains supplementary tables. Supplementary table-1

List of abbreviations

asRNA: antisense transcript; iNOS: inducible nitric oxide synthase; NO: nitric oxide; IL-1β: interleukin 1β; 3'UTR: 3'-untranslated region; ARE: AU-rich element; NATRE: natural antisense transcript-targeted regulation; RT: reverse transcription; PCR: polymerase chain reaction; IL-23A: interleukin 23, α subunit; CCL: chemokine C-C motif ligand; CX3CL1: chemokine C-X,3-C motif ligand 1; CXCL1: chemokine C-X-C motif ligand 1;
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