Age-related expression analysis of mouse liver nuclear protein binding to 3'-untranslated region of *Period2* gene

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**Abstract** In mammals, both circadian rhythm and aging play important roles in regulating time-dependent homeostasis. We previously discovered an age-related increase element binding protein, hnRNP A3, which binds to the 3'-untranslated region (UTR) of blood coagulation factor IX (FIX). Here, we describe other members of this protein family, hnRNP C and hnRNP H, which bind to the 3'-UTR of the mouse circadian clock gene *Period 2 (mPer2)*. RNA electrophoretic mobility shift assays using a 32P-labeled *Per2* RNA probe coupled with two-dimensional gel electrophoresis followed by MALDI-TOF/MS peptide mass fingerprint analysis was used to analyze these proteins. Western blotting suggested that the total expression of these proteins in mouse liver cell nuclei does not increase with age. Two-dimensional gel electrophoresis analysis of age-related protein expression showed that many isoforms of these proteins exist in the liver and that each protein exhibits a complex age-related expression pattern. These results suggest that many isoforms of proteins are regulated by different aging systems and that many age regulation systems function in the liver.

**Keywords** *Per2* · hnRNP · Circadian rhythm · Aging · Homeostasis

**Introduction**

In homeostatic systems, stable gene expression is maintained during aging via several factors, such as the age-related stability element (ASE)/age-related increase element (AIE) [1–4]. The AIE, originally identified in the *hFIX* gene, contains a 102-bp stretch of dinucleotide repeats (AT, GT, and CA) that have the potential to form distinct stem-loop (sl) [1–4] RNA structures (FIX-AIE RNA) in the 3'-untranslated region (3'-UTR) after the gene is transcribed [5]. We recently reported that heterogeneous nuclear ribonucleoprotein (hnRNP) A3 binds to the AIE and plays an important role in age-related gene expression [4]. HnRNP A3 is a member of the hnRNP family of proteins. These proteins, which are generally known to bind RNA, are most abundant in the nucleus of mammalian cells, where they play critical roles in the synthesis and processing of precursor RNAs in the nucleus and mRNA...
transportation from the nucleus to the cytosol. HnRNP A3 recognizes the overall three-dimensional sl structures formed by AIE RNA rather than specific nucleoside sequences [4].

In homeostatic systems, cell-based negative transcriptional feedback loop regulation is important for circadian expression of clock genes that are responsible for daily oscillations in physiology and behavior. In addition, posttranscriptional regulation via the 3'-UTR of genes appears to be important in the expression of circadian clock genes [6, 7]. HnRNP R, Q, and L reportedly bind to the 3'-UTR of serotonin N-acetyltransferase [arylalkylamine N-acetyltransferase (AANAT)], which is the key enzyme in melatonin synthesis and is regulated by the circadian system, resulting in rhythmic AANAT mRNA degradation [6]. LARK reportedly interacts with the 3'-UTR of the Per1 gene, regulating its expression in a posttranscriptional manner, recognizing the sl structure rather than the specific RNA sequence [7].

In the present study, we found that the hairpin sequence in the 3'-UTR of Per2 has the potential to form an sl RNA structure (hereafter mPer2 sl) after the gene is transcribed. In addition, we identified two mouse liver nuclear proteins, hnRNP H and C, which bind specifically to the 3'-UTR of Per2. We also describe the age-related expression profiles of these proteins.

**Materials and methods**

**Construction of mPer2 sl-RNA probes**

32P-labeled mPer2 sl-RNA was prepared by in vitro transcription for 2 h at 37 °C using a T7-MEGAscript high-yield kit (Ambion, Austin, TX, USA) with a template DNA fragment of mPer2 sl (39 bp). Template DNA fragments were generated using the sequence 5'-TAA TACgACTcACTATAgg TACACTggCTTTTTTgTTT TAggAAAAACAAAAACAA-3' (19 bp for the T7 promoter + 39 bp for mPer2), which corresponds to the sl region spanning nucleotides (nts) 4006–4043 of the mPer2 gene (Genbank accession number: AF036893).

Transcription using the mPer2 sl DNA fragments thus generated was carried out in a reaction mixture (final volume of 20 μL) composed of 2 μL of 10× transcription buffer provided in the Ambion kit, 2 μL each of 75 mM ATP, GTP, and CTP, 4 μL of α-[32P]UTP (800 Ci/mmol; GE Healthcare, Buckinghamshire, UK), 1 μg of mPer2 template DNA, and 2 μL of T7 MEGAscript enzyme mix. After 2 h incubation at 37 °C, the reaction mixture was added with 1 μL of RNase-free DNase solution, incubated for 15 min at 37 °C and subjected to electrophoresis using a 6% polyacrylamide urea gel to remove template DNA fragments and unincorporated nucleotides. The gel was then exposed to an X-ray film for 10 s, and gel areas containing radioactivity were precisely located by matching with the autoradiogram and excised. The gel pieces recovered were then incubated in a probe elution buffer (Ambion) at 37 °C overnight. Non-radioactive mPer2 sl-RNA probes were prepared in a similar manner by using non-radioactive nucleosides in transcription reaction.

**UV cross-linking and electrophoresis of RNA/protein complexes**

Electrophoretic mobility shift assays (EMSAs) were carried out as previously described (Fig. S1) [4]. Nuclear extracts (NEs) were prepared from liver tissues of 6-month-old C57BL/6 mice (Charles River Laboratories, Yokohama, Japan). Animal care and use procedures were reviewed and approved by the committee for animal experimentation of the National Institute of Advanced Industrial Science and Technology (AIST) (Permission #36-07-009) and were performed in accordance with the institutional guidelines of the Committee for Animal Experimentation in the AIST. Some animal work was performed in accordance with the Hokkaido University Guidelines for the Care and Use of Laboratory Animals, under permission #14-0059 from the Hokkaido University Committee for Animal Experimentation.

**Identification of liver nuclear proteins bound to the mPer2 sl-RNA probe**

Identification of liver nuclear proteins bound to the mPer2 sl-RNA probe was carried out as previously described [4]. We showed a flowchart of the identification of mPer2 sl-RNA binding protein in supplemental Fig. 1. RNA electrophoretic mobility shift assays using a 32P-labeled Per2 RNA probe coupled with two-dimensional gel electrophoresis followed by MALDI-TOF/MS peptide mass fingerprint analysis was used to analyze these proteins.

**Western blotting of liver nuclear proteins bound to the mPer2 sl-RNA probe**

Liver NEs protein samples were prepared from C57BL/6J mice at various ages, as previously described [4]. Proteins were solubilized in 20 μL of SDS loading buffer. After adjusting the protein concentration using the BCA method, samples were subjected to 10% SDS-PAGE. Western blotting analyses were carried out according to standard methods, using a horseradish peroxidase–conjugated antirabbit antibody for protein detection with the ECL assay system (GE Healthcare). Band intensity was measured using a Fujix Bio-imaging analyzer LAS 1000 v.3.4X software (Fujifilm, Tokyo, Japan). Mouse anti-hnRNP F/H
We found potential hairpin sequences in the 3′-UTR of the Per2 gene (Fig. 1a, b). GENETYX-MAC analysis predicted that the secondary structure of this region would form a sl structure (Fig. 1c). The 3′-UTRs of the rat Per2 (rPer2) (Genbank accession number: NM031678) and human Per2 (hPer2) (Genbank accession number: NM022817) genes also contain potential hairpin sequences (rPer2 sl and hPer2 sl) between nts 3974 and 3994 in rPer2 (5′-TTCACTgCT TCTTTTgTTTTAgAAAAAAACAAACAC-3′) and between nts 4110 and 4147 in hPer2 (5′-CATgTTgCT TTTTTgTTTTAgAAAAAAACAAACATA-3′) (Fig. 1a). The mPer2, rPer2, and hPer2 sl structures are located after a stop codon about 100 bp in length (the mPer2, rPer2, and hPer2 stop codons are located at nts 3916–3918, 3876–3878, and 4003–4005, respectively). The mPer2 sl showed 86.8 and 90.6 % homology to rPer2 sl and hPer2 sl, respectively.

As shown in Fig. 1b, a 32P-labeled mPer2 sl-RNA probe was prepared from the region spanning nts 4006 and 4043 of the mPer2 gene. RNase treatment of the 32P-labeled mPer2 sl-RNA probe cross-linked by UV treatment to mouse liver nuclear proteins revealed some bands corresponding to RNA probe/nuclear protein complexes (Fig. 1d). Of these, two bands were identified with shifted mobility and for which the intensity increased with increasing amounts of added NE (Fig. 1d, lanes 2–5). These two bands were observed at approximately 37 and 50 kDa (Fig. 1d, lanes 2–5). These shifted bands effectively competed with 10-, 50-, and 100-fold excess amounts of non-radiolabeled (cold) mPer2 sl-RNA probe (Fig. 1e, lanes 2–4), indicating that they were specifically generated by the 32P-labeled mPer2 sl-RNA probe.

Identification of liver nuclear proteins bound to the mPer2 sl-RNA probe

An amount of 32P-labeled mPer2 sl-RNA/protein complex sufficient for preparative solution-phase IEF was obtained by repeating the RNA EMSA procedures with the 32P-labeled mPer2 sl-RNA probe, the UV-irradiation and RNase digestion of the extracted 32P-labeled mPer2 sl-RNA probe/protein complex, SDS-PAGE separation, and subsequent excision of the radioactive gel area and extraction of the treated complex using electrodution, as previously described [4]. Analytical 2DE of the complex after concentration and autoradiography showed that preparative solution-phase IEF efficiently concentrated most of the 32P-labeled mPer2 sl-RNA/protein complex within the pl 4–5 zone, consistent with a previous report (Fig. S1) [4]. The 2DE and subsequent autoradiographic analyses of the pooled and concentrated solutions of the pl 3–4.6 and pl 4.6–5.4 zones identified distinct multiple radioactive gel spots, including a major spot between approximately 40 and 50 kDa that exhibited the most intense radioactivity (data not shown). MALDI-TOF/MS and PMF analyses of the proteins extracted from this major radioactive gel spot.
at approximately 40 kDa identified hnRNP C as the protein component, with a Mascot score of 270 (Expect: 8.3e-023; Sequence Coverage: 46 %) (Table 1, Fig. S3). Similar analyses of 32P-labeled mPer2 sl-RNA probe/nuclear protein complexes at approximately 50 kDa identified hnRNP H2 and H1, with Mascot significant scores of 171 (Expect: 6.6e-013; Sequence Coverage: 56 %) and 159 (Expect: 1e-011; Sequence Coverage: 65 %), respectively (Table 1, Fig. S4). No other proteins were identified by the MALDI-TOF/MS and PMF analyses (Table 1).

**Age-related expression of mPer2 sl-RNA–binding proteins in the liver**

Using monoclonal anti-hnRNP F/H and anti-hnRNP C1/C2 antibodies, western blot analysis was carried out with liver NEs obtained from 1-, 3-, 8-, 16-, and 21-month-old C57BL/6 mice (Figs. 2a, 2S). HnRNP H and its variants are closely related to hnRNP F [10]. The major hnRNP H protein bands, at approximately 50 kDa, were identified according to a previous report [10]. The major hnRNP C1/
C2 protein bands, at approximately 40 kDa, were also identified [11]. Expression of HnRNP F/H peaked at 3 months of age and remained stable thereafter (Fig. 2b). Expression of HnRNP C1/C2 peaked at 8 months of age (Fig. 2b). The expression of these proteins differed from that of both HuR (an AU-rich RNA domain binding protein that showed an age-stable expression pattern; Fig. 2a, b) and hnRNP A3, the expression of which has been shown to increase with age [4].

**2DE analysis of age-related expression hnRNP C1/C2 and hnRNP H1/H2 in the liver**

To examine the age-related expression of hnRNP C1/C2 and hnRNP H1/H2 in detail, we analyzed the liver nuclear proteins of 1-, 3-, 6-, 12-, 18-, 21-, and 24-month-old mice using 2DE combined with MALDI-TOF/MS (Fig. 2c–f). All 2DE gel spots (total number of spots, 3113; number of single spots, 2557; number of mixed spots, 556) of proteins from 3-month-old mice were examined using MALDI-TOF/MS analysis. HnRNP C1/C2 was identified in 7 single spots and 3 mixed spots (Table 2; Figs. 2c, S6). Many spots were distributed between pl 5 and pl 6 and between 37 and 39 kDa. The age-related expression of 4 single spots (A2319, A2427, A2432, and A2433) is illustrated in Fig. 2d. Spots B6819, B8328, and B9112 spots in Table 2 were disregarded due to their very different molecular weight from the band corresponding to the RNA/nuclear protein complex (Fig. 1e) or because the intensity of the spot was very low. Spot A2432 exhibited the highest expression intensity, followed by spots A2319, A2433, and A2427, in that order. The expression of these proteins tended to decline after 18 months of age (Fig. 2d).

HnRNP H1 was identified in 3 single spots and 4 mixed spots, whereas hnRNP H2 was identified in 1 single spot and 4 mixed spots (Table 2; Figs. 2e, S7, S8). The expression of both hnRNP H1 and H2 was relatively lower than that of both hnRNP C1 and C2 (Fig. 2d, f). The age-related expression of 4 single spots (A1520, A4520, A6405, and A514) is illustrated in Fig. 2f. The expression patterns of these proteins were unique. Spot N6405, identified as hnRNP H1, peaked at 12 months of age. After 21 months, the expression of spot N4520 decreased. The expression of spot N6514 remained constant. The expression of spot A1520, identified as hnRNP H2, decreased after 3 months of age.

**Discussion**

In the present study, we found that the proteins hnRNP C and hnRNP H bind to the 3'-UTR region of the Per2 gene, which has the potential to form distinct sl RNA structures after transcription. Both hnRNP C and hnRNP H are members of the hnRNP family, which is composed of more than 20 proteins, including hnRNP A1, A2, and A3 [10, 11]. As previously reported, we found that hnRNP A3 binds to the AIE in 3'-UTR of the Factor IX gene and recognizes the overall three-dimensional sl structures of AIE RNAs rather than specific nucleoside sequences [4]. In contrast to hnRNP A3, neither hnRNP C nor hnRNP H exhibited age-related differences in expression in the liver in this study. These results indicate that hnRNP C and hnRNP H play different roles than hnRNP A3 in regulating gene expression in the liver.

Similar to other hnRNP family proteins, various protein modifications in addition to alternative splicings might give rise to many hnRNP C and hnRNP H proteins detected in 2DE [12–18]. Supplementary Fig. 3 show 2DE analysis of the mouse liver NEs along the age axis and spots originated from hnRNP A3 protein. HnRNP A3 is regulated by various types of modifications including phosphorylation [4, 17, 18], methylation [19, 20] and sumoylation [21]. Our data show HnRNP A3 proteins received modification including the phosphorylation at its Ser359 and the level of this phosphorylation increased with age. In contrast, the number of spots of hnRNP C and hnRNP H proteins were stable along the age axis (Fig. 2). Unfortunately, we could not detect any phosphorylation sites of HnRNP C or hnRNP H in the present experiment by MS analysis. Further studies are needed to analyze the modification sites of HnRNP C and hnRNP H proteins.

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**Table 1** Mouse liver nuclear proteins that bound to the mPer2 sl-RNA probe

| Accession   | Mass   | Score | Description                                      |
|-------------|--------|-------|-------------------------------------------------|
| Upper band  |        |       |                                                 |
| gi 9845253  | 49533  | 171   | Heterogeneous nuclear ribonucleoprotein H2 (Mus musculus) |
| gi 10946928 | 49454  | 159   | Heterogeneous nuclear ribonucleoprotein H1 (Mus musculus) |
| gi 56206900 | 51470  | 154   | Heterogeneous nuclear ribonucleoprotein H1 (Mus musculus) |
| Lower band  |        |       |                                                 |
| gi 8393544  | 34421  | 270   | Heterogeneous nuclear ribonucleoprotein C (Mus musculus) |
| gi 13435678 | 32257  | 268   | HnRcp protein (Mus musculus)                     |

MALDI-TOF/MS PMF analysis of mPer2 sl-RNA binding proteins
The present study showed that there are a number of isoforms of hnRNP C and hnRNP H, and that these isoforms exhibit complex age-related expression patterns. Our data suggest that many protein isoforms are regulated differentially during aging and that many mechanisms function in the liver to regulate protein expression during aging.
HnRNPs also reportedly play important roles in the post-translational regulation of circadian clock gene expression. In the cryptochrome (Cry) gene, hnRNP D binds to the middle part of the 3′-UTR, which contains destabilizing cis-acting elements and contributes to Cry1 mRNA turnover and modulation of the circadian rhythm [22]. AANAT expression is also post-transcriptionally regulated by hnRNP R, Q, and L via the 3′-UTR of the gene [23]. The expression of hnRNP U in the mouse SCN (suprachiasmatic nucleus), in which the master circadian pacemaker is located, shows circadian rhythm [24]. Using the full-length 3′-UTR, Woo et al. showed that hnRNP I in the cytoplasm of CHO-K1 cells binds to the CU-rich portion of 3′-UTR of the mouse Per2 gene and that many RNA-binding proteins interact with Per2 mRNA, including two proteins of approximately 40 and 50 kDa [25]. These two proteins may correspond to hnRNP C and hnRNP H, which were identified in the present study (Fig. 1d).

Table 2 Identification of hnRNP C1/C2, H1, and H2 from all 2DE gel spots

| SSP number | pI  | Mr   | Spot quantity | MS hit score | Spot info | MS hit score in the mixture |
|------------|-----|------|---------------|--------------|-----------|----------------------------|
| **HnRNP C1/C2** |
| A2319 | 5.2402 | 37,099.7 | 518 | 64 | Single | 0 |
| A2328 | 5.0951 | 36,932.8 | 371.8 | 129 | Mix | 58 |
| A2427 | 5.0982 | 38,763.6 | 179.5 | 63 | Single | 0 |
| A2431 | 5.0805 | 37,737.2 | 886.2 | 239 | Mix | 44 |
| A2432 | 5.1581 | 37,737.2 | 1071.3 | 63 | Single | 0 |
| A2433 | 5.1672 | 38,817.7 | 265.2 | 57 | Single | 0 |
| A4328 | 5.7305 | 35,918.0 | 49.9 | 126 | Mix | 67 |
| B6819 | 9.1533 | 82,564.2 | 16.8 | 46 | Single | 0 |
| **HnRNP H1** |
| N4520 | 5.9448 | 53,089.3 | 53.5 | 119 | Single | 0 |
| N5502 | 5.9950 | 56,267.0 | 925.8 | 342 | Mix | 94 |
| N5523 | 6.3721 | 52,715.2 | 187.6 | 295 | Mix | 58 |
| N5525 | 6.0081 | 55,342.4 | 0 | 222 | Mix | 85 |
| N6405 | 6.4684 | 46,614.3 | 57.8 | 133 | Single | 0 |
| N6422 | 6.4560 | 48,411.4 | 210.7 | 274 | Mix | 60 |
| N6514 | 6.8543 | 56,426.1 | 142.1 | 182 | Single | 0 |
| **HnRNP H2** |
| A1520 | 4.9649 | 47,840.4 | 349.7 | 196 | Single | 0 |
| N3521 | 5.8593 | 56,011.9 | 200.2 | 298 | Mix | 119 |
| N4511 | 5.9372 | 55,463.9 | 209.8 | 223 | Mix | 140 |
| N4526 | 5.9962 | 54,836.2 | 97.2 | 167 | Mix | 111 |
| N6407 | 6.5283 | 49,276.6 | 338.1 | 278 | Mix | 68 |

All spots for samples from 3-month-old mice were identified by MALDI-TOF/MS PMF analysis (total number of spots, 3113; number of single spots, 2557; number of mixed spots, 556). Spots identified as hnRNP C1/C2, H1, and H2 are listed. A total of 10, 7, and 5 spots were identified as hnRNP C1/C2, hnRNP H1, and hnRNP H2, respectively, among all 2DE spots analyzed. The locations of these spots are shown in Fig. 2c, e and Figs. S6, S7, and S8.
regulation via the 3′-UTR of genes appears to be an important system for maintaining circadian clock gene expression patterns in a number of species. CCTR and CHLAMY1 in *Gonyaulax polyedra* and *Chlamydomonas*, AtGRP7 in *Arabidopsis*, and LARK in *Drosophila*, are all reportedly involved in regulating the circadian rhythm of translation [26–29]. After transcription, *Per1* forms a sl RNA structure in its 3′-UTR. In mammals, the RNA-binding protein LARK interacts with the 3′-UTR of *Per1* and post-transcriptionally regulates *Per1* expression [7].

In this study, we could not detect the spot arising from the PER2 protein in our 2DE/MALDI-TOF/MS analyses of age-related mouse liver nuclear protein expression. To obtain a better understanding of the relationship between age and PER2 expression, it is important that future studies identify which isoform of hnRNP C1/C2 or H1/H2 binds to the *Per2* sl structure.

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Conflict of interest None of the authors have any conflict of interest.

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