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ORIGINAL PAPER

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 ^{32}P-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 Period 2 · hnRNP · Circadian rhythm · Aging · Homeostasis

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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

^{32}P-labeled *mPer2* sl-RNA was prepared by in vitro transcription for 2 h at 37 °C using a T7-MEGAshortscript 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 TACACTggCTTTTTTTgTTT TAggAAAAACAAAAAACAA-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-[^{32}P]UTP (800 Ci/mmol; GE Healthcare, Buckinghamshire, UK), 1 μg of *mPer2* template DNA, and 2 μL of T7 MEGAshortscript 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 ^{32}P-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 anti-rabbit 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

(mouse monoclonal; 1G11; ImmuQuest, Cleveland, UK), anti-hnRNP C1/C2 (mouse monoclonal; 4F4; ImmuQuest), and anti-mouse HuR (mouse monoclonal; 19F12; Molecular Probes, Eugene, OR, USA) antibodies were used.

Two-dimensional gel electrophoresis (2DE) analysis of age-dependent expression of hnRNP C/C2 and hnRNP H1/H2 in the liver

Mice (C57BL/6J mice) were maintained for at least 1 week on a 12 h light/12 h dark (LD) cycle with lights on 0600–1800 hours. The sampling of mouse liver was performed during 0930–1200 hours. Liver NEs (300 μg each) prepared from 1-, 3-, 6-, 12-, 18-, 21-, and 24-month-old mice ($n = 20$ mice at each age) were subjected to isoelectric focusing (IEF) at pH 4–7 (acidic), pH 5–8 (neutral), and pH 6–11 (basic) using 24-cm Immobiline Dry Strips (Bio-Rad, Hercules, CA, USA), followed by SDS-PAGE on pre-cast 10–16 % gradient polyacrylamide gels (Bio-Rad). Each sampling was done once and each gel was used for analysis. The gels were then stained with colloidal Coomassie Brilliant Blue (CBB) and scanned using a GS-800 scanner (Bio-Rad). Protein spots were analyzed using PDQuest software (v.7.1, Bio-Rad). The gel images were normalized and the protein spots were excised, destained in 50 mM ammonium bicarbonate/50 % acetonitrile, and dried in a vacuum concentrator (Savant, Holbook, NY, USA). The dried gel pieces were then rehydrated with 5 μL of a solution of 20 ng/L trypsin in 10 mM ammonium bicarbonate and incubated at 30 °C overnight. Mass spectrometric analysis of the tryptic peptides was carried out as described previously [4]. All spots were subjected to matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF/MS) peptide mass fingerprint (PMF) analysis on an Axima® CFR MALDI-TOF mass spectrometer (Kratos, Manchester, UK/Shimazu Biotech, Kyoto, Japan). PMF data were searched against the National Center for Biotechnology Information (NCBI) database using the internet-available program Mascot (Matrix Science, London, UK). Each matched protein spot was assigned a unique sample spot protein (SSP) number prefixed by A (acidic), N (neutral), or B (basic) in the PDQuest software. Analysis of age-related expression of hnRNP C1/C2 and hnRNP H1/H2 was carried out using an age-related mouse liver nuclear protein database that we constructed (unpublished) [8, 9].

Results

RNA-EMSAs of *mPer2* sl-RNA with mouse liver NE

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 TCTTTTgTTTTAgAAAAAAAAACAAAACAC-3′) and between nts 4110 and 4147 in *hPer2* (5′-CATgTTgCT TTTTTTgTTTTAgAAAAAAAAACAACATA-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 ^{32}P-labeled *mPer2* sl-RNA probe was prepared from the region spanning nts 4006 and 4043 of the *mPer2* gene. RNase treatment of the ^{32}P-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 ^{32}P-labeled *mPer2* sl-RNA probe.

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

An amount of ^{32}P-labeled *mPer2* sl-RNA/protein complex sufficient for preparative solution-phase IEF was obtained by repeating the RNA EMSA procedures with the ^{32}P-labeled *mPer2* sl-RNA probe, the UV-irradiation and RNase digestion of the extracted ^{32}P-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 electroelution, as previously described [4]. Analytical 2DE of the complex after concentration and autoradiography showed that preparative solution-phase IEF efficiently concentrated most of the ^{32}P-labeled *mPer2* sl-RNA/protein complex within the pI 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 pI 3–4.6 and pI 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

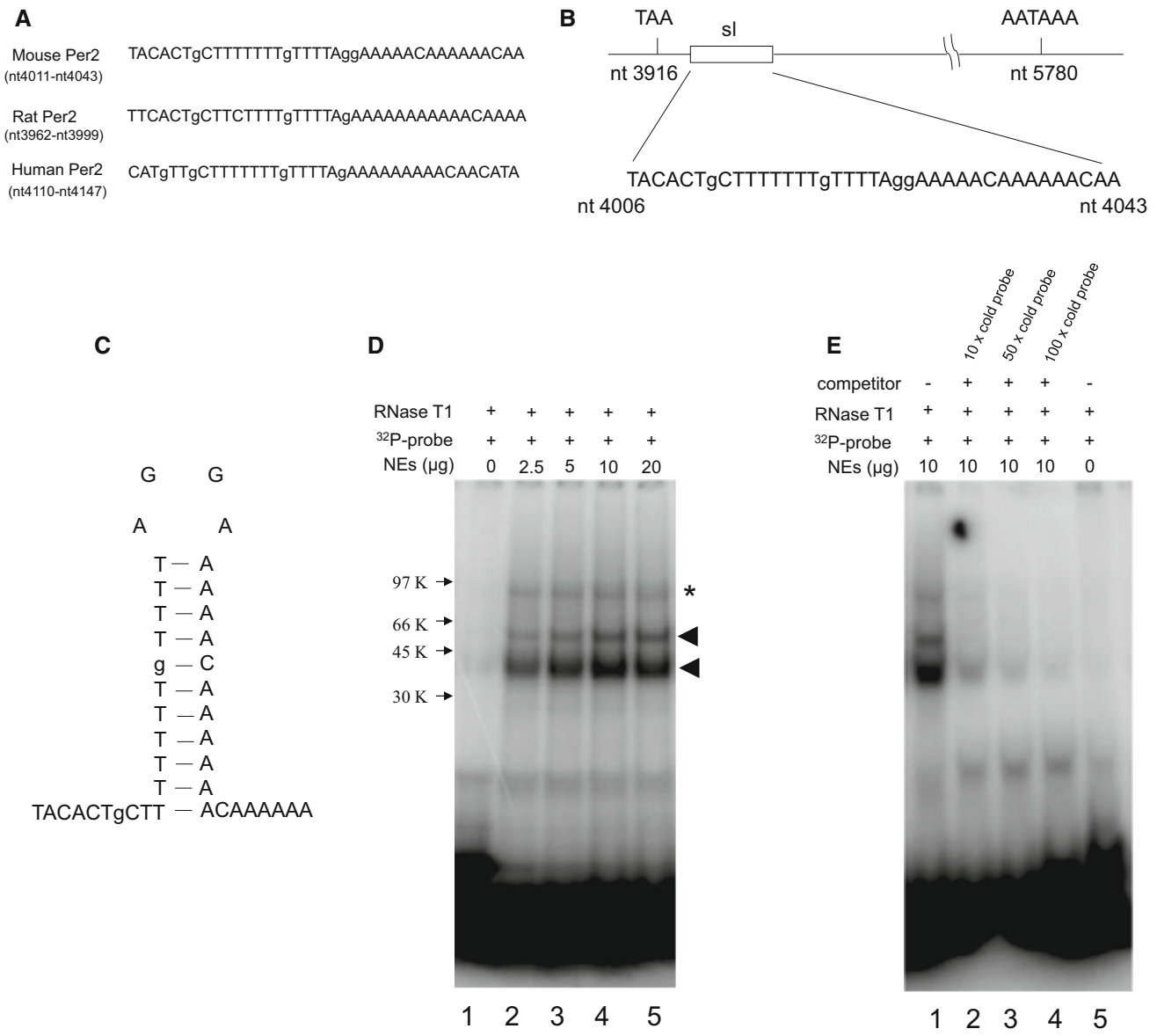


Fig. 1 RNA-EMSA of ^{32}P-labeled *mPer2* sl-RNA with mouse liver NE. **a** Purine–pyridine sequence in the 3′-UTR of the *Per2* gene. **b** Location of the potential hairpin sequence in the 3′-UTR (starting at nucleotide 4006) of the *mPer2* gene. **c** The potential 3′-UTR hairpin (starting at nucleotide 4006) in the *mPer2* gene. **d** RNA-EMSA of the ^{32}P-labeled *mPer2* sl-RNA probe followed by UV cross-linking and RNase treatment. RNA-EMSA of ^{32}P-labeled *mPer2* sl-RNA with NE. *Arrowheads* indicate the *mPer2* sl-RNA probe–protein complexes. *Lanes 1–5* contain ^{32}P-labeled *mPer2* sl-RNA and RNase T1. *Lane 1* no NE; *lanes 2–4* with liver NE. *Asterisk* indicates that band intensity did not increase with increasing amount of NE added. **e** Competitive RNA-EMSA of ^{32}P-labeled *mPer2* sl-RNA with NE. *Lanes1–5* contain ^{32}P-labeled *mPer2* sl-RNA and RNase T1. *Lanes 2–4* contain unlabeled *mPer2* sl-RNA and NE. *Lane 5* contains no NE

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 ^{32}P-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/

Table 1 Mouse liver nuclear proteins that bound to the m*Per2* 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	Hnrpc protein (*Mus musculus*)

MALDI-TOF/MS PMF analysis of *mPer2* sl-RNA binding proteins

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

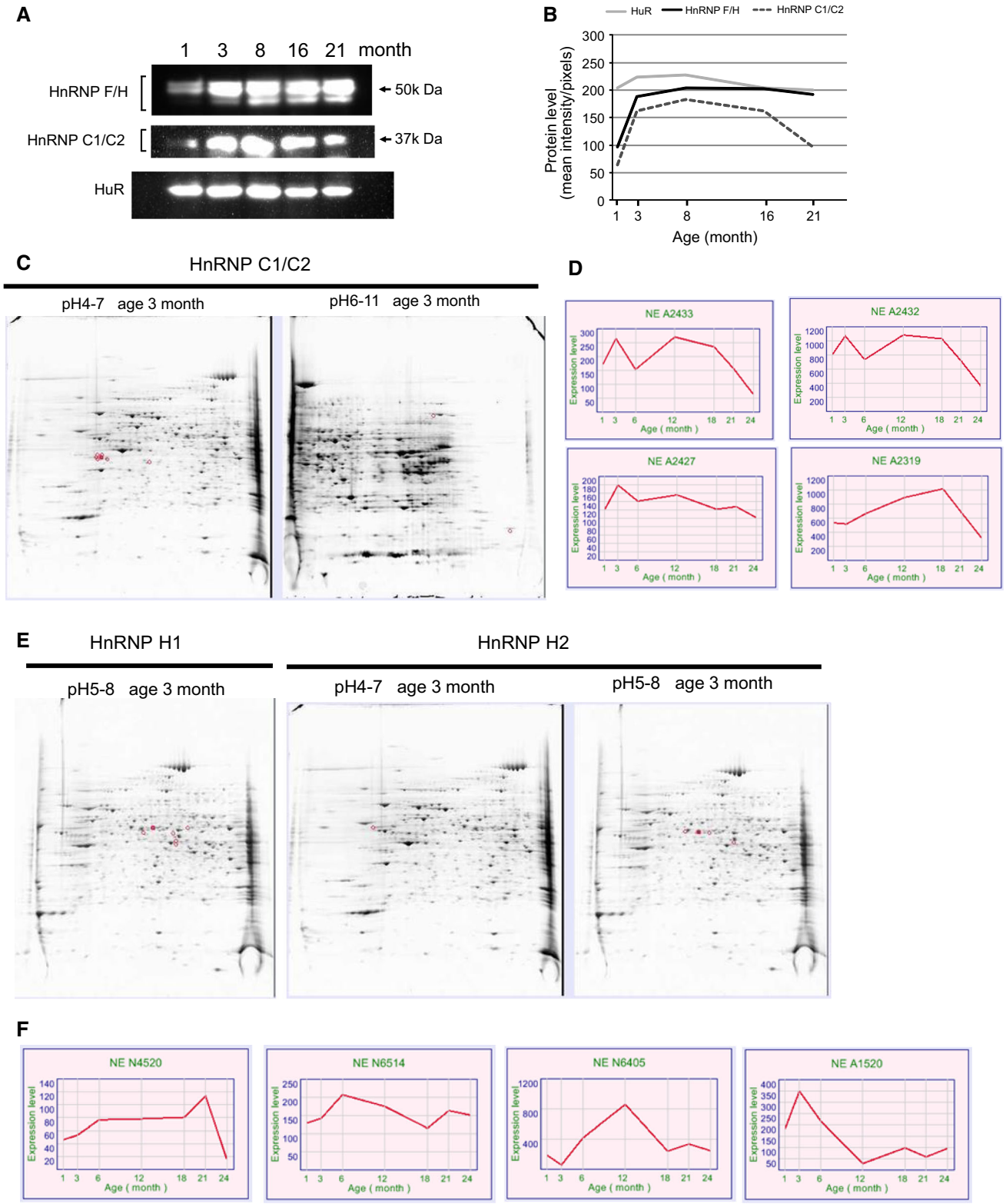
To examine the age-related expression of hnRNP C1/C2 and hnRNP H1/H2 in detail, we analyzed the liver nuclear proteins of 1-, 3-, 6-, 12-, 18-, 21-, and 24-month-old mice using 2DE combined with MALDI-TOF/MS (Fig. 2c–f). All 2DE gel spots (total number of spots, 3113; number of single spots, 2557; number 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 pI 5 and pI 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 spots 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 Ser^{359} 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.



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.

◀**Fig. 2** Age-related expression of *mPer2* sl-RNA–binding proteins in the liver. **a** Western blotting analysis of age-related hnRNP H and hnRNP C expression using monoclonal antibodies and 10 μg of male mouse liver NE. Molecular weight is indicated on the *right*, and the age of the animals is shown at the *top*. **b** Graphical illustration of the patterns of hnRNP C1/C2 and hnRNP H/F expression as shown in (**a**). Protein expression is shown as the total mean intensity of all bands at each age. **c** 2DE analysis of age-related expression of hnRNP C1/C2 in the liver. Representative images of 2DE gels for 3-month-old mice. CBB stained spots are shown in the images from immobilized pH 4–7, 5–8, and 6–11 gradient IEF gels and 10–16 % gradient SDS-PAGE gels. Spots identified as hnRNP C1/C2 are shown with *red circles*. **d** Age-related expression profiles of spots identified as hnRNP C1/C2. **e** 2DE analysis of age-related expression of hnRNP H1/H2 in the liver. Spots identified as hnRNP H1/H2 are shown with a *red circle*. The location of each spot is shown with *red circle* in Figs. S6, S7 and S8. **f** Age-related expression profiles of spots identified as hnRNP H1/H2. Spot N5525 in Table 2 is not shown because the intensity of spot was very low. Mouse liver NEs were sampled between 0930 and 1200 hours (lights on between 0600 and 1800 hours) (color figure online)

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). Post-transcriptional

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

SSP number	pIr	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
B8328	9.5825	35,397.8	0	49	Single	0
B9112	10.0112	17,936.2	66.1	66	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	2029.8	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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