Identification of the mammalian homologues of the *Drosophila timeless* gene, *Timeless1*

Nobuya Koike, Akiko Hida, Rika Numano, Matsumi Hirose, Yoshiuki Sakaki, Hajime Tei*

Human Genome Center, Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

Received 24 November 1998

Abstract We have identified novel mammalian homologues of a *Drosophila* clock gene, *timeless*, and designated them as human *TIMELESS1* (h*TIM1*) and mouse *Timeless1* (m*Tim1*), respectively. These genes were mapped by FISH to chromosomal regions 12q12-13 in human and 10D3 in mouse. The deduced amino acid sequences of hTim1 and mTim1 proteins were 1208 and 1197 amino acids in length and shared 83% identity. Northern blot analysis identified a single transcript of 4.5 kb expressed widely in many tissues examined. Unlike the *Drosophila* counterpart, the levels of the m*Tim1* transcript exhibited no prominent circadian oscillation in the mouse brain.

Key words: Circadian rhythm; Timeless homologue; Mammalian clock gene

1. Introduction

Many physiological and behavioral processes exhibit circadian rhythms. Although the cellular mechanisms controlling circadian rhythms differ between *Drosophila* [1] and mammals [2,3], several homologous clock genes have been found in both. In *Drosophila*, oscillation in the levels of the products of *period* (*per*) [4,5] and *timeless* (*tim*) [6–9] is responsible for the circadian rhythms of locomotor activity. These oscillations are maintained by the function of *Jerk* (*Jrk*) [10], *cycle* (*cyc*) [11], *per* [4], *tim* [12], and *double-time* (*dbt*) [13]. Mutations in either one of these genes abolish both the oscillations of the *per* and *tim* products and behavioral rhythms. The expressions of *per* and *tim* are activated by the function of the heterodimer of Jrk and Cyc, and feedback inhibited by their own products, which close the cycle of the circadian expressions of both genes [14]. Thus the autoregulation mechanism appears to be responsible in turn for the zenith and nadir phases of the circadian oscillation of *per* and *tim*. As in the case of *Drosophila*, the expression of a mouse homologue of *Drosophila per*, m*Per1*, is induced by the concerted action of mClock and mBmal1 [15,16]. However, since none of the mammalian homologues of *tim* and *dbt* have been identified, the molecular mechanisms regulating the circadian expression of *Per1* [17,18] remain unknown. In this report, we describe the identification of the human and mouse homologues of *Drosophila tim* (h*TIM1* and m*Tim1*, respectively). The mammalian Tim1 proteins contain five homologous regions to *Drosophila* Tim (dTim) including the NLS (Nuclear Localization Signal) and the regions required for binding to Per. The structural resemblance suggests mammalian Tim1 may function as a molecular component within a circadian clock. Strong expression of m*Tim1* is detected in the mammalian central clock, the suprachiasmatic nucleus (SCN) of the mouse brain. However, unlike the *Drosophila* counterpart, the levels of the m*Tim1* transcript exhibit no prominent circadian oscillation in the mouse brain.

2. Materials and methods

2.1. Molecular cloning

Human or mouse brain poly(A)$^+$ RNA (1 μg) were used for each reverse transcription reaction with a Superscript preamplification system (Gibco-BRL Life technologies). The primers used for amplification were PF01e (TTAGCTCAGGAGCGGAGTGCAG) and PR01e (CTCCTGATAGGCCTTCAGAGCC), and MF01r (TGCTACAGCTGGGCTGGGAGG) and MR01e (TTCCACATCCTTGTGGGCGAGG) for the human and mouse brain cDNA, respectively. Reactions were performed in a total volume of 50 μl containing specific primer sets (10 pmol each) and Ex Taq polymerase (Takara Shuzo). PCR cycling was initiated by a 1-min denaturation followed by 30 cycles of denaturation (94°C for 45 s), annealing and extension (68°C for 2 min) with a final extension of 3 min at 72°C. Approximately 10^6 clones of a λDR2 5′-stretch cDNA library of human brain and cerebellum (Clontech Laboratories) and 2×10^6 clones of a λgt10 5′-stretch-plus cDNA library of mouse brain (Stratagene) were screened, with a h*TIM1* EST clone as a probe. Positively hybridizing plaques were purified, the cDNA inserts were rescued as phagemids according the manufacturer's instructions. 5′- and 3′-RACE were performed by the Marathon RACE Kit (Clontech Laboratories). The products were subcloned into a T-vector, pT7blue(R) (Novagen), and sequenced. Subsequently, the h*TIM1* and m*Tim1* cDNA fragments synthesized by RT-PCR were directly sequenced to eliminate misincorporation during RACE. Sequence reactions were performed with a dye-terminator cycle-sequencing-ready reaction kit (Perkin-Elmer). Each sample was electrophoresed with an ABI 373S sequencer (Perkin-Elmer). Homology search was performed by the blastn, blastp, blastx and tblastn programs using non-redundant nucleotide (nr-nt), non-redundant amino acid (nr-aa) and dbEST databases.

2.2. Northern hybridization

Northern blots (Clontech Laboratories) containing 2 μg of human and mouse poly(A)$^+$ RNA from multiple tissues were hybridized and washed as described above. Filters were exposed to Imaging-Plates (Fuji Photo Film), and analyzed by the BAS2000A model Bioimage-analyzer (Fuji Photo Film).

2.3. In situ hybridization

Male BALB/c mice (JAP), 8–10 weeks old, were used. After 2 weeks of adaptation to the standard 12 h light:12 h dark (LD) cycle, half of the mice were transferred to an environment of constant darkness (DD) and kept there for 2 days, the rest being kept under LD. Serial coronal sections (40 μm thick) of the mouse brain were made using a cryostat, and quantitative in situ hybridization of m*Tim1* mRNA was performed as described previously [19].

*Corresponding author. Fax: (81) (3) 5449-5445.
E-mail: tei@ims.u-tokyo.ac.jp

The sequences for h*TIM1* and m*Tim1* are deposited in the DDBJ database, accession numbers AB015597 and AB015598, respectively.

PII: S0014-5793(98)01597-X

3. Results

3.1. Cloning of the human and mouse Tim1 cDNA

A homology search based on the amino acid sequence of *Drosophila* Tim (dTim) was performed by the tblastn program against the dbEST database. Two human (zv01d06.r1 and zv01d06.s1) and one mouse (mm77b08.r1) ESTs showed significant homology to a region of d*tim* encoding the domain required for binding to *Drosophila* Per (dPer) [20,21]. Screening of human and mouse brain cDNA libraries with the human EST clone zv01d06 of approximately 600 bp was performed in conjunction with 5′- and 3′-RACE, resulting in identification of the human and mouse cDNA clones of 4354 bp and 4417 bp, respectively. We designated these human and mouse cDNAs as h*TIM1* and m*Tim1*, respectively. The h*TIM1* and m*Tim1* cDNA sequences possess open reading frames encoding polypeptides of 1208 and 1197 amino acid residues, with predicted molecular weights of 138 403 Da and 137 501 Da (Fig. 1). The amino acid sequences of hTim1 and mTim1 share 83% identity (Fig. 2). These genes have been mapped by FISH to chromosomal regions 12q12-13 in human and 10D3 in mouse, syntenic loci between the two animals (data not shown), indicating that the two genes are orthologues in these species.

Significant homology between the two mammalian Tim1 and *Drosophila* Tim proteins is confined to five stretches (Figs. 1 and 2): (A) residues 1–229 of both hTim1 and mTim1, (B) residues 313–325 of both hTim1 and mTim1, (C) residues 342–521 of both hTim1 and mTim1, (D) residues 693–754 of hTim1 and 687–748 of mTim1, and (E) residues 1040–1083 of hTim1 and 1037–1080 of mTim1. The extent of homology in these five stretches is 53%, 67%, 45%, 50% and 50%, respectively (Fig. 1). Although the functions of the aforementioned regions A and E in dTim have not been determined, it should be noted that these regions are also highly conserved among Tim proteins from different *Drosophila* species. Region A shares 93% homology between *D. melanogaster* and *D. virilis* [22], while region E shares 95% homology (Fig. 2). Furthermore, *tim*^{r} (*ritsu*), a *tim* allele of *D. melanogaster* discovered because of its prolonged circadian period in locomotor activity, has a single amino acid substitution in a residue completely conserved in region E of all Tim homologues (Matsumoto and Tanimura, personal communication; Fig. 2). These results indicate the functional importance of region E. Although point mutations in dTim have not been located in regions B, C and D, biochemical studies have indicated that these regions also represent important roles [21]. Region B contains a signal for nuclear localization of dTim (a basic amino acid cluster) and a binding domain to the PAS-A domain of dPer, and regions C and D correspond to a binding domain of dTim to the cytoplasmic localization domain (CLD) of dPer (Fig. 1). In mammalian Tims, the amino acid stretch homologous to this binding domain is split into two regions, C and D (Figs. 1 and 2). The most distinct structural difference between mammalian Tim1 and *Drosophila* Tim resides in the C-terminal region. In the Tim of *D. melanogaster*, the CLD (Cytoplasmic Localization Domain) has been revealed in the C-terminus (positions 1237–1398) by biochemical analysis [21]. However, no CLD except two short homologous stretches of an acidic amino acid cluster is found in mammalian Tim. An identical 3′ sequence for both h*TIM1* and m*Tim1* cDNAs has been obtained by both 3′-RACE and cDNA screening methods (Fig. 2). In addition, the lengths of the cDNAs correlate well with those of the respective transcripts as observed in Northern blot analysis (Figs. 1 and 3; and described below). Thus, it is unlikely that the absence of the homologous C-terminal region in mammalian Tims is an artifact caused by truncation of the mammalian *Tim* cDNAs during cloning.

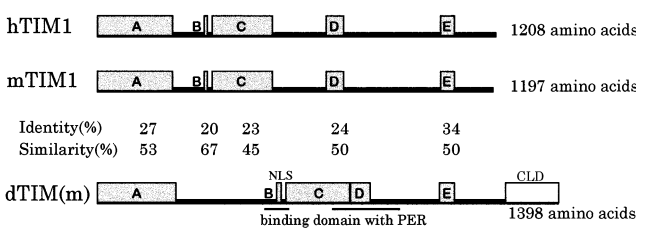

Fig. 1. Schematic comparisons of the Tim family. Homologous regions are indicated by shaded boxes (A–E). The sequences corresponding to CLD in dTim (m) are boxed, and the binding domain with Per is underlined.

3.2. Tissue expression of hTIM1 and mTim1

Expression patterns of h*TIM1* and m*Tim1* were examined by Northern hybridization. h*TIM1* and m*Tim1* transcripts of approximately 4.5 kb were detected in all of the human and mouse tissues including brain, heart, lung, liver, skeletal muscle, kidney, placenta, pancreas, spleen, and testis (Fig. 3-1 and -2). In addition, the m*Tim1* transcript was expressed throughout the entire mouse embryonic stages examined (Fig. 3-3). The wide spatial expression is a common feature of potential clock genes such as *Per1* [17,18], *Clock* [23,24], and *Bmal1* [25].

3.3. Time dependence of mTim1 expression in the mouse brain

Expression of m*Tim1* in coronal sections of the mouse brain was examined by in situ hybridization. Strong signals were detected in the suprachiasmatic nucleus (SCN) and pars tuberalis, and moderate signals in the cingulate cortex, periventricular part of the caudate putamen, and granular cell layer of the cerebellum, although weak signals were observed in the cerebral cortex and thalamic nuclei (Fig. 4-1). The results strongly suggest that Tim1 colocalizes with Per1 [17,18], Clock [23,24], and Bmal1 [25] within the mammalian clock cells. Expression of the *Drosophila tim* and *per* genes exhibited circadian oscillation and transcription of both genes was under the autonomous control of dTim and dPer [4,6,14]. These facts prompted us to examine the expression of m*Tim1* in the brain under both 12 h light:12 h dark (LD) and constant dark (DD) conditions by a quantitative in situ hybridization method. Both in LD and DD, none of the tissues in the brain including the SCN displayed clear circadian oscillation in m*Tim1* expression (Fig. 4-2 and -3). These results indicate that the transcriptional regulation of m*Tim1* in the SCN is quite different from that of m*Per1*, for which a robust circadian oscillation has been identified [17].

4. Discussion

Our structural analysis indicates that hTim1 and mTim1 are closely related to each other and share homology with several regions of *Drosophila* Tim. hTim1 and mTim1 share five ho-

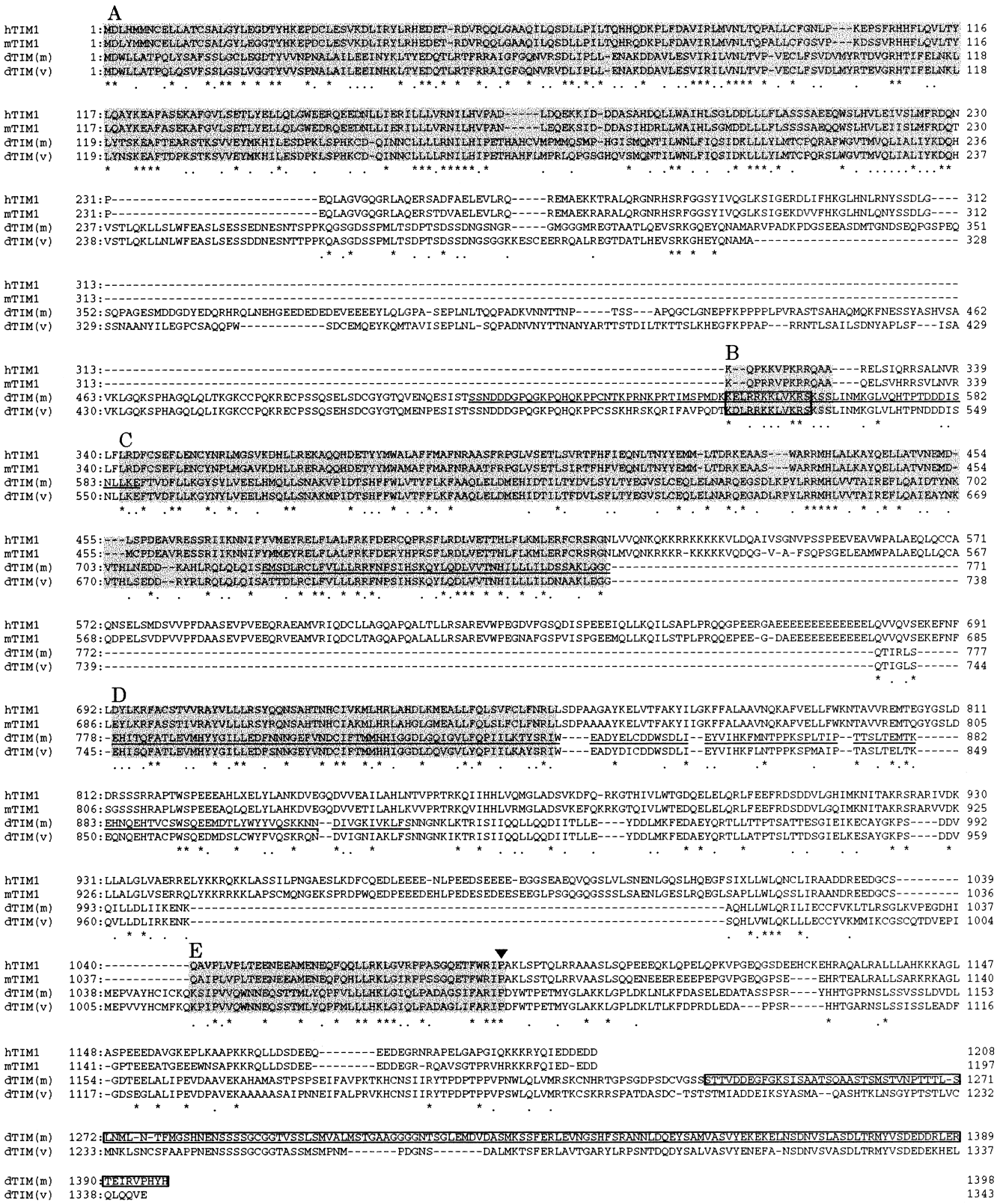

Fig. 2. Amino acid sequence comparisons of the Tim family. The homologous regions from A to E are indicated as described in Fig. 1. The sequences corresponding to NLS, CLD in dTim (m) are boxed, and the binding domain to Per is underlined. The arrowhead indicates the position of the amino acid substitution in Tim^{r}. Amino acid identity and similarity between hTim1, mTim1, dTim (m) (*D. melanogaster*) and dTim (v) (*D. virilis*) are indicated by asterisks and periods below the dTim (v) sequence, respectively.

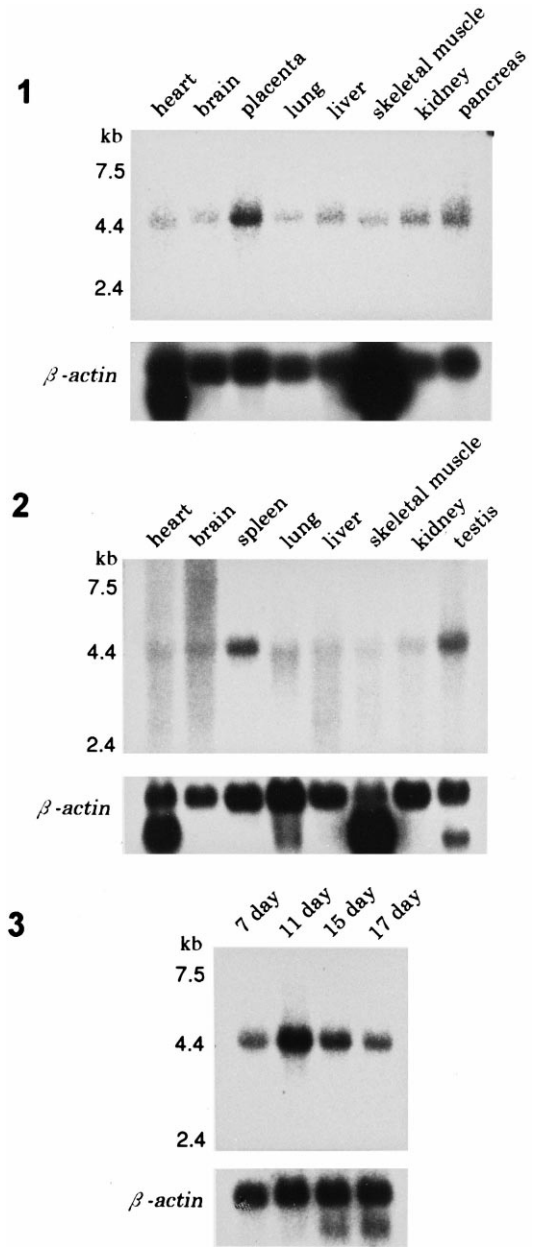

Fig. 3. Expression of h*TIM1* and m*Tim1* analyzed by Northern blot analysis. The filters (1, human; 2, mouse; and 3, mouse embryo) were probed with species-specific *Tim1*, and β-*actin*.

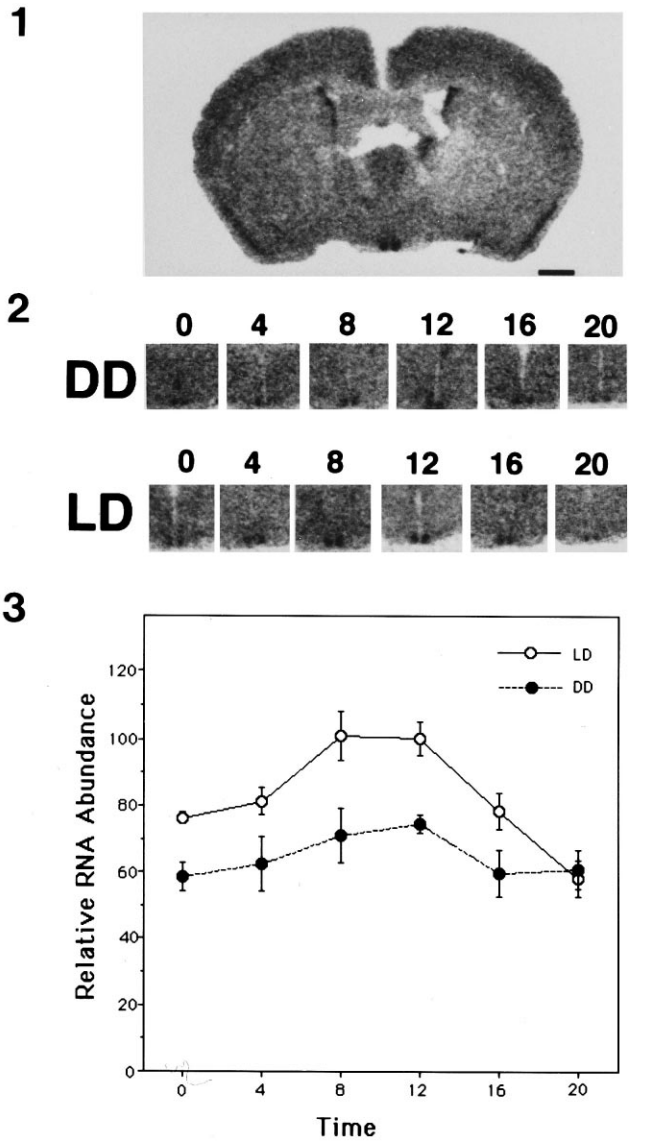

Fig. 4. Expression of h*TIM1* and m*Tim1* analyzed by in situ hybridization. 1: In situ hybridization of m*Tim1* in the mouse forebrain during the daytime of LD condition (ZT8). Scale bars = 1 mm. 2: Representative in situ hybridization autoradiograms showing SCN at ZT0, 4, 8, 12, 16 and 20 in LD and CT0, 4, 8, 12, 16 and 20 in DD. 3: Rhythmic expression of m*Tim1* in LD (open circle) and DD conditions (closed circle) by quantitative in situ hybridization. Each value is the mean ± S.E.M. ($n = 5$). Plots of RNA cycling set mean peak value to 100.

mologous regions with dTim. The homologous regions include NLS, the region required for binding to Per in addition to the domain in which a genetic mutation (*tim*^{r}) has been mapped (Fig. 2). Two other homologous regions outside of these regions in the Tim family suggest the conserved function of the regions to be elucidated. The most distinct structural difference between mammalian Tim1 and *Drosophila* Tim resides in the C-terminal region. However, comparisons between the Tims of *D. melanogaster* and *D. virilis* [23] have revealed that the C-terminal sequence is more divergent than other homologous regions (Fig. 2). Homology between the Tim C-termini of *D. melanogaster* and *D. virilis* is about 60%, in contrast to those of the other homologous regions described above: 93%, 100%, 96%, 97%, and 95%, in regions A, B, C, D, and E, respectively. Despite the absence of the entire CLD, homologous stretches containing an acidic amino acid cluster is found in Tims of human (positions 1174–1181, and 1203–1208), mouse (positions 1166–1172, and 1193–1197) and *Drosophila* (positions 1382–1385). The results indicate that the C-terminal region is not vital for Tim to achieve clock function, or only a short region within the C-termini of Tim is required for the CLD function.

Wide distribution of mammalian *Tim1* expression is a common feature of the candidates of the circadian clock genes. Even though the clock that generates behavioral rhythms resides in a small cluster of neurons in the brain, autonomous circadian clocks have recently been identified in many non-neuronal cells in both *Drosophila* [26] and mammals [27,28].

These observations suggest that common molecular components are involved in the circadian clocks in both the brain and other peripheral tissues.

The structural resemblance of mammalian Tim1 suggests its role as a circadian clock component in the mammalian system, although the molecular mechanisms whereby *Tim1* regulates circadian rhythms remain to be elucidated. One intriguing possibility is that Tim1 binds to Per1, resulting in the inhibition of *Per1* expression. However, m*Tim1* exhibited no clear circadian expression in the mouse SCN (Fig. 4), in contrast to the robust circadian oscillation of d*tim* in the fly brain [6]. This result suggests a difference between the utilization of m*Tim1* and d*tim* in the circadian clocks of the two animals. There is also a precedent for species variation in the molecular details of the regulation of the clock genes, as the dynamics of *Per* regulation in silkmoth brain are different from those in *Drosophila* [29]. Establishment of genetic mutant mice for m*Tim1* and biochemical analysis of the interactions between the products of *Tim1*, *Per1*, *-2*, and *-3*, *Clock*, and *Bmal1*, in addition to the analysis of the *Per2* and *-3* promoters are expected to provide further insight into the molecular mechanisms of m*Tim1* function in the mammalian clock system.

Acknowledgements: We thank Drs. H. Okamura, S. Miyake, S. Taguchi, S. Takekida, S. Onishi, S. Sumi (Kobe University) for sharing the data of in situ hybridization, Drs. J. Inazawa and M. Hirai for mapping hTIM1 and mTim1, respectively, Drs. M. Hattori and T. Ito for discussion, Drs. D. Yamamoto, and K. Ui-Tei for comments and discussions on the manuscript. This work was partially supported by research grants from the Japanese Ministry of Education, Science, Sports and Culture, and the Japanese Ministry of Health and Welfare.

References

[1] Ewer, J., Frisch, B., Hamblen-Coyle, M.J., Rosbash, M. and Hall, J.C. (1992) J. Neurosci. 12, 3321–3349.
[2] Moore, R.Y. and Eichler, V.B. (1972) Brain Res. 42, 201–216.
[3] Stephen, F.K. and Zucker, I. (1972) Proc. Natl. Acad. Sci. USA 69, 1583–1586.
[4] Hardin, P.E., Hall, J.C. and Rosbash, M. (1990) Nature 343, 536–540.
[5] Zerr, D.M., Hall, J.C., Rosbash, M. and Siwicki, K.K. (1990) J. Neurosci. 10, 2749–2762.
[6] Sehgal, A., Rothenfluh-Hilfiker, A., Hunter-Ensor, M., Chen, Y., Myers, M.P. and Young, M.W. (1995) Science 270, 808–810.
[7] Zeng, H., Qian, Z., Myers, M.P. and Rosbash, M. (1995) Nature 380, 129–135.
[8] Myers, M.P., Wager-Smith, K., Rothenfluh-Hilfiker, A. and Young, M.W. (1996) Science 271, 1736–1740.
[9] Hunter-Ensor, M., Ousley, A. and Sehgal, A. (1996) Cell 84, 677–685.
[10] Allada, R., White, N.E., So, W.V., Hall, J.C. and Rosbash, M. (1998) Cell 93, 791–804.
[11] Rutila, J.E., Suri, V., Le, M., So, W.V., Rosbash, M. and Hall, J.C. (1998) Cell 93, 805–814.
[12] Sehgal, A., Price, J.L., Man, B. and Young, M.W. (1994) Science 263, 1603–1606.
[13] Price, J.L., Blau, J., Rothenfluh, A., Abodeely, M., Kloss, B. and Young, M.W. (1998) Cell 94, 83–95.
[14] Darlington, T.K., Wager-Smith, K., Ceriani, M.F., Staknis, D., Gekakis, N., Steeves, T.D.L., Weitz, C.J., Takahashi, J.S. and Kay, S.A. (1998) Science 280, 1599–1603.
[15] Gekakis, N., Staknis, D., Nguyen, H.B., Davis, F.C., Wilsbacher, L.D., King, D.P., Takahashi, J.S. and Weitz, C.J. (1998) Science 280, 1564–1569.
[16] Hogenesch, J.B., Gu, Y.Z., Jain, S. and Bradfield, C.A. (1998) Proc. Natl. Acad. Sci. USA 95, 5474–5479.
[17] Tei, H., Okamura, H., Shigeyoshi, Y., Fukuhara, C., Ozawa, R., Hirose, M. and Sakaki, Y. (1997) Nature 389, 512–516.
[18] Sun, Z.S., Albrecht, U., Zhuchenko, O., Bailey, J., Eichele, G. and Lee, C.C. (1997) Cell 90, 1003–1011.
[19] Shigeyoshi, Y., Taguchi, K., Yamamoto, S., Takekida, S., Yan, L., Tei, H., Moriya, T., Shibata, S., Loros, J.J., Dunlap, J.C. and Okamura, H. (1997) Cell 91, 1043–1053.
[20] Gekakis, N., Saez, L., Delahaye-Brown, A.M., Myers, M.P., Sehgal, A., Young, M.W. and Weitz, C.J. (1995) Science 270, 811–815.
[21] Saez, L. and Young, M.W. (1996) Neuron 17, 911–920.
[22] Myers, M.P., Rothenfluh, A., Chang, M. and Young, M.W. (1997) Nucleic Acids Res. 25, 4710–4714.
[23] King, D.P., Zhao, Y., Sangoram, A.M., Wilsbacher, L.D., Tanaka, M., Antoch, M.P., Steeves, T.D., Vitaterna, M.H., Kornhauser, J.M., Lowrey, P.L., Turek, F.W. and Takahashi, J.S. (1997) Cell 89, 641–653.
[24] Antoch, M.P., Song, E.J., Chang, A.M., Vitaterna, M.H., Zhao, Y., Wilsbacher, L.D., Sangoram, A.M., King, D.P., Pinto, L.H. and Takahashi, J.S. (1997) Cell 89, 655–667.
[25] Ikeda, M. and Nomura, M. (1997) Biochem. Biophys. Res. Commun. 233, 258–264.
[26] Plautz, J.D., Kaneko, M., Hall, J.C. and Kay, S.A. (1997) Science 278, 1632–1635.
[27] Balsalobre, A., Damiola, F. and Schibler, U. (1998) Cell 93, 929–937.
[28] Zylka, M.J., Shearman, L.P., Weaver, D.R. and Reppert, S.M. (1998) Neuron 20, 1103–1110.
[29] Sauman, I. and Reppert, S.M. (1996) Neuron 17, 889–900.