

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

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Abstract We have identified novel mammalian homologues of a *Drosophila* clock gene, *timeless*, and designated them as human *TIMELESS1* (hTIM1) and mouse *Timeless1* (mTim1), 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 mTim1 transcript exhibited no prominent circadian oscillation in the mouse brain.

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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*, *mPer1*, 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 (hTIM1 and mTim1, 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 mTim1 is detected in the mammalian central clock, the suprachiasmatic nucleus (SCN) of the mouse brain. However, unlike the *Drosophila* counterpart, the levels of the mTim1 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 (TTAGCTCAGGAGCGGAGTGACAG) and PR01e (CTCCTGATAGGCCTTCAGAGCC), and MF01r (TGCTACAGCTGGGCTGGGAGG) and MR01e (TTCCACATCCTGTGGGCGAGG) 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 10⁶ clones of a λDR2 5'-stretch cDNA library of human brain and cerebellum (Clontech Laboratories) and 2×10⁶ clones of a λgt10 5'-stretch-plus cDNA library of mouse brain (Stratagene) were screened, with a hTIM1 EST clone as a probe. Positively hybridizing plaques were purified, the cDNA inserts were rescued as phagemids according to 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 hTIM1 and mTim1 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 Bio-imaging-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 mTim1 mRNA was performed as described previously [19].

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The sequences for hTIM1 and mTim1 are deposited in the DDBJ database, accession numbers AB015597 and AB015598, respectively.

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 *dtim* 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 *hTIM1* and *mTim1*, respectively. The *hTIM1* and *mTim1* 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

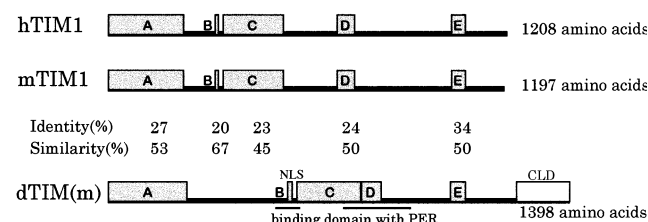


Fig. 1. Schematic comparisons of the Tim family. Homologous regions are indicated by shaded boxes (A–E). The sequences corresponding to CLD in dTim (m) are boxed, and the binding domain with Per is underlined.

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 *hTIM1* and *mTim1* 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.

3.2. Tissue expression of *hTIM1* and *mTim1*

Expression patterns of *hTIM1* and *mTim1* were examined by Northern hybridization. *hTIM1* and *mTim1* 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 *mTim1* 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 *mTim1* 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 *mTim1* 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 *mTim1* expression (Fig. 4-2 and -3). These results indicate that the transcriptional regulation of *mTim1* in the SCN is quite different from that of *mPer1*, 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-

A	
hTIM1	1: MDLHMNCELLATCSALGYLEGGTYNKEPDCLESVKDLIRYLRHEDET--RDVROQLGAAQILQSDLLPILTQHRODKPLFDVAVIRLMVNLTPALLCPGNLP---KEPSFRHHFLQVUTY 116
mTIM1	1: MDLYMNCCELLATCSALGYLEGGTYNKEPDCLESVKDLIRYLRHEDET--RDVROQLGAAQILQSDLLPILTQHRODKPLFDVAVIRLMVNLTPALLCPGSPV---KDSVFRHHFLQVUTY 116
dTIM (m)	1: MDWLATPOLYSAPSSGLCEGGTYVUNPNALALEEINYKLTVEEDQTLRTPFRAAIGFGQNVVDLIPLL--ENAKDDAVLESVIRILVNLVFP--VECLFSDVMYRTVGRTTYFELNKL 118
dTIM (v)	1: MDWLATPOLQSVSSGLSGVGGTYVUNPNALALEEINHKLTVEEDQTLRTPFRAAIGFGQNVVDLIPLL--ENAKDDAVLESVIRILVNLVFP--VECLFSDVMYRTVEVGRHTTYFELNKL 118
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hTIM1	117: LQAYKEAFASEKAFQVLSPTLYELLQGLWEEQEDNLLIERILLVNRNLHVPA---LDQEKKID--DDASANDQLLWAIHLSGLDLLLLFLASSAEQNSLHVLEIVSLMFRDQN 230
mTIM1	117: LQAYKEAFASEKAFQVLSPTLYELLQGLWEEQEDNLLIERILLVNRNLHVPA---LEQEKSID--DDASIHDRLLWAIHLSGMDDLFLSSSSAQNSLHVLEITSLMFRDQT 230
dTIM (m)	119: LYTSTKFAFTFARSTKSVVEYMKHILESDEPKLSPHKCD--QINNCILLRNILHIEPTHANCVMPPMGSMP--HGTSMONTILNLFQISIDKLLLYLMTCPQAPNGVTMVLIALIYKDOH 236
dTIM (v)	119: LYNKFAFTDPKSTKSVVEYMKHILESDEPKLSPHKCD--QINNCILLRNILHIEPTHANFLMPRIQPGSGHOVSMONTILNLFQISIDKLLLYLMTCPQASISLGVMTVQLIALIYKDOH 237
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hTIM1	231: P-----EQLAGVGQGRLAQERSADFAEVLVRQ-----REMAEKKTRALQGRNHRFRGGSYIVQGLKSIGERDLIFHKGLHNLNRNYSDDLQ----- 312
mTIM1	231: P-----EQLAGVGQGRLAQERSTDVAEVLVRQ-----REMAEKRRARALQGRNHRFRGGSYIVQGLKSIGEDVVFHKGHLNQLNYSDDLQ----- 312
dTIM (m)	237: VSTLQKLLSLWFASLESSESDNESNTSPPKQSGSDSPMLTSDPTSDSDNGSNR-----GMGGMRREGTAATLQEVSRKGQEQYNAMARVPADKPDGSEASDMDTGNDSBQPGSPQ 351
dTIM (v)	238: VSTLQKLLNLWFASLESSESDNESNTTPPKQSGSDSPMLTSDPTSDSDNGSGGKESCEERRQALREGTDATLHEVSRKGHEYNAMA----- 328
* * * * *	
hTIM1	313: ----- 462
mTIM1	313: ----- 429
dTIM (m)	352: SQPAGESMDGDYEDQRRQLNEHGEDEDEVEVEEYLQGLPA--SEPLNLTQPADKVNNTNP-----TSS---APQGLGNEFFKPPPLPVRASTSAHAQMQKFNESSYASHVSA 462
dTIM (v)	329: SSNAANYILEGPCSAQPFW-----SDCEMQEYKQMTAVISEPLNL--SQPADNVNNTNANYARTTSTDILTCTSLKHGEGFPAP---RRNTLSAILSDNYAPLSF---ISA 429
* * * * *	
B	
hTIM1	313: -----K---QPKVKPKRQAA-----RELSIQRRSALNVR 339
mTIM1	313: -----K---QPRVPEKPKRQAA-----QELS VHRHSLVLR 339
dTIM (m)	463: VKLGQKSPHAGQLQTLKGCKCPQKRECPSSQSELSDCGYGTQVENQESISTSSNDDGPGQKPOHQPCPNKPTIMSEMDKELRKKLKKRQSSSLINMKGLVQHPPTDDDIS 582
dTIM (v)	430: VKLGQKSPHAGQLQLIKGCCPKQKRECPSSQSEHSDCGYGTQVENPESISTSSNDDGPGQKPOHQPCSSKRRSKQRIFAVPQDTKDLRKKLKKRQSSSLINMKGLVLTPTDDDIS 549
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C	
hTIM1	340: LFLRDFCSEFLNENYRNLGMSVQKDHLLREKAQOHDETYVMWALFFMAFNRAASFRLVSELTISVTRPHFIEQNLTNYIEM--LTDKEAAS---WARRMHLAKAYQELLATVNEMD 454
mTIM1	340: LFLRDFCSEFLNENYRNLGMSVQKDHLLREKAQOHDETYVMWAMAFMAFNRAASFRLVSELTISRTHFVEQNLTNYIEM--LTDKEAAS---WARRMHLAKAYQELLATVNEMD 454
dTIM (m)	583: NLLKEFTVDFLKGYSYLVVELHLMQLLSNAKVPIDTSHFVLTVTYFLKFAAQLELMEHIDITLTVDVLSYLYTVEGVSLCQLELNARQEGSDLKPYLRMHVLTATREPLQAITDYNK 702
dTIM (v)	550: NLLKEFTVDFLKGYSYLVVELHLMQLLSNAKVPIDTSHFVLTVTYFLKFAAQLELMEHIDITLTVDVLSYLYTVEGVSLCQLELNARQEGADLRPYLRMHVLTATREPLQAITDYNK 669
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hTIM1	455: ---LSPDEAVRESSRIKNNIFVMEYRELFLALRKFEDRCQPRSLRDLVETHTLFLKMLERFCRSRGNLVQNKQKKRKKKKKVLQDAIVSGNVFSSPEEVAWPALAEQLQCCA 571
mTIM1	455: ---MCPDEAVRESSRIKNNIFVMEYRELFLALRKFEDRYHFKSFLRDLVETHTLFLKMLERFCRSRGNLMVQNKRRK--KKKKVQDQG--V-A-FSQPSGEAMWPALAEQLQCCA 567
dTIM (m)	703: VTHLNEDD---KAHLRQLQLQISEMSDLRCLFVLLRRFNFSIHSKQYLODLVVTNHLILLDSSAKLGG----- 771
dTIM (v)	670: VTHLSEDD---RYRLRQLQLQISATDRLCLFVLLRRFNFSIHSKQYLODLVVTNHLILLDSSAKLGG----- 738
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hTIM1	572: QNSELSDMSVVPFDDAASEVPVEEQRAEAMVRIQDCLLAGAQPAQALTLRSAREVWPEBGDVFSGSDISPEEIEQLLKQILSAPLPRQGGPEERGAEEEEEEEEEEELQVVQVSEKEFNF 691
mTIM1	568: QDPELSVDVPVFPDDAASEVPVEEQRAEAMVRIQDCLTAGAQPAQALALLRSAREVWPEBNAFGSPVISPEGEMLLKQILSTPLPRQGEPEE--G-DAEEEEEEEEEEELQVVQVSEKEFNF 685
dTIM (m)	772: -----QTIRLS----- 777
dTIM (v)	739: -----QTIGLS----- 744
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D	
hTIM1	692: LDYLKRFACSTVIRAYVLLRSYQNSAHTNHCIVMKHLRLAHLKMEALLPQLSFLCPFLNRLSDPAAGAYKELVTFAKYILGKFFALAAVQKAFVELLFWKNTAVVREMGYQSGSLD 811
mTIM1	686: LEYLKRFACSTVIRAYVLLRSYQNSAHTNHCIAKMLHLRLAHLKMEALLPQLSFLCPFLNRLSDPAAAYKELVTFAKYIIGKFFALAAVQKAFVELLFWKNTAVVREMGYQSGSLD 805
dTIM (m)	778: ---EHTQFATLEVMHYVYGLLEDFPNNGEFVNDCEFTMMHIGGLDGGIQLVLFQITLKTYSRIN---EADYELCDDWSDLI---EYVIHKFMNTPPKSPLTIP---TTSLETMTK--- 882
dTIM (v)	745: ---EHTQFATLEVMHYVYGLLEDFPNNGEFVNDCEFTMMHIGGLDGGIQLVLFQITLKTYSRIN---EADYDICDDWSDLI---EYVIHKFMNTPPKSPLTIP---TASLTBLTK--- 849
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hTIM1	812: DRSSSRAPRTPSPPEEAHLXELYLANKDVEGQDVVEAILAHLNLTVPTRKQIHHVLVQMLGADSVKDFQ--RKGTHIVLWTDGQLELQRLFEFRDSDVVLGHIMKNITAKRSRARIVDK 930
mTIM1	806: SGSSSRAPRTPSPPEEAHLXELYLANKDVEGQDVVEITLAHLKVVPTRKQVHHVLVQMLGADSVKDFQ--RKGTHIVLWTDGQLELQRLFEFRDSDVVLGHIMKNITAKRSRARIVDK 925
dTIM (m)	883: EHNQHTVCSWSQGEEMDTLYWYVYQSKNN--DIVGKIVKLFSSNGKLNKTRISIIQQLLQDITLLE---YDDLKMFEDAEOYQRTLLTTPTSATPESGIEKECAYGKFS---DDV 992
dTIM (v)	850: EQNQHTACFPWSQGEEMDSCWYFVQSKRN--DVIGNIAKLFSSNGKLNKTRISIIQQLLQDITLLE---YDDLKMFEDAEOYQRTLLTTPTSATPESGIEKESAYGKFS---DDV 959
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hTIM1	931: LLAIGLVAERRELYKKRQKLASSILPNGAESLKDFQEDLEEE--NLPEEDSEEEE--EGGSEAEVQVGSVLNENLQSLHQEGFSIXLLWLQNLIRAAADREEDGCS----- 1039
mTIM1	926: LLAIGLVSERRQYKKRRKLAPSCMONGEKSPRDPQWEDPEEDELPEDESEDESEGLPSGQGGSSSLAENLQESLRQEGLSAPLLWLQSLIRAAANDREEDGCS----- 1036
dTIM (m)	993: QTLDLI IKENK-----AQHLLWLQRLIECCFVKLTLSRSLKVPBGDHI 1037
dTIM (v)	960: QVLLDLIRKENK-----SQHLLWLQRLLECCYVKMKIKCSQGTDEPEI 1004
* * * * *	
E	
hTIM1	1040: -----QAVPLVPLTEENBEAMENQFOHLLRKLGIKRPSSQGETFWIRPAKLSPTQLRRAAASLSQPEEQKLOPELQPKVPGEQGSDEEHCKEHRQAALRALLAHKKKAGL 1147
mTIM1	1037: -----QATPLVPLTEENBEAMENQFOHLLRKLGIKRPSSQGETFWIRPAKLSSTQLRRVAASLSQGENEEREPEEPGVGEGQGPSE---EHRTEALRALLSARKKAGL 1140
dTIM (m)	1038: MEPVAYHCICKOKSIPVQVNNQSTIMLYQPFVLLHKLGIQLPADAGSIFARIEDFTVTPETMYGLAKKLGLDKLNLKFDASELEDATASSPSR---YHHTGPRNLSVSSSLDVL 1153
dTIM (v)	1005: MEPVYVHCMPKOKPIPVQVNNQSTIMLYQPFVLLHKLGIQLPADAGSIFARIEDFTVTPETMYGLAKKLGLDKLNLKFDPRDEDA---PPSR---HHTGARNLSLSSISLEADF 1116
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hTIM1	1148: ASPEEEDAVGKEPLKAPKKRQLLDSDEQ-----EEDGRNRAPELAGPAGIKKKRYQIEDDED 1208
mTIM1	1141: -GPTTEEATGEEENWAPKKRQLLDSDEE-----EDDEGR--RQAVSGTPRVHKKRQIED--EDD 1197
dTIM (m)	1154: -GDTEELALPEVDAAVEKAHAMASTPSPSEIFAVPKTKHCNSIIRYTPDPTFPVNVWLQVLVMSKCNHRTGSPGDFSDCVGSSSTTVDDCEGCKSISAASTQAASTSMSTVNPITTL--S 1271
dTIM (v)	1117: -GDSEGLALPEVDFAVEKAAAAASAI PNNEIFALPRVHKHCNSIIRYTPDPTFPVNVWLQVLVMTKCKRRSPATDASDC--TSTSTMIADDEIKSYASMA---QASHTKLSNGYPTSTLV 1232
* * * * *	
dTIM (m)	1272: <u>LNMLN-TFPGSHNENSSSSGCGGTSSLSMVALMSGAAGGGGNTSGLEMDVDAASKKSFERLEVNGSHFSRANNLDQESANVASVYERKEELNSDNVSLASDLTRMYVSDDEDRLE</u> 1389
dTIM (v)	1233: MNKLSNCSFAAPPNNENSSSGCGGTASSMSMPNM-----PDGNS-----DALMKTSEFLAVTGARYLRPSNTDQDYSALVASVYENFA--NSDNVSVASDLTRMYVSDDEDEKHEL 1337
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dTIM (m)	1390: <u>TEIRVPHYV</u> 1398
dTIM (v)	1338: QLQQVE 1343

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.

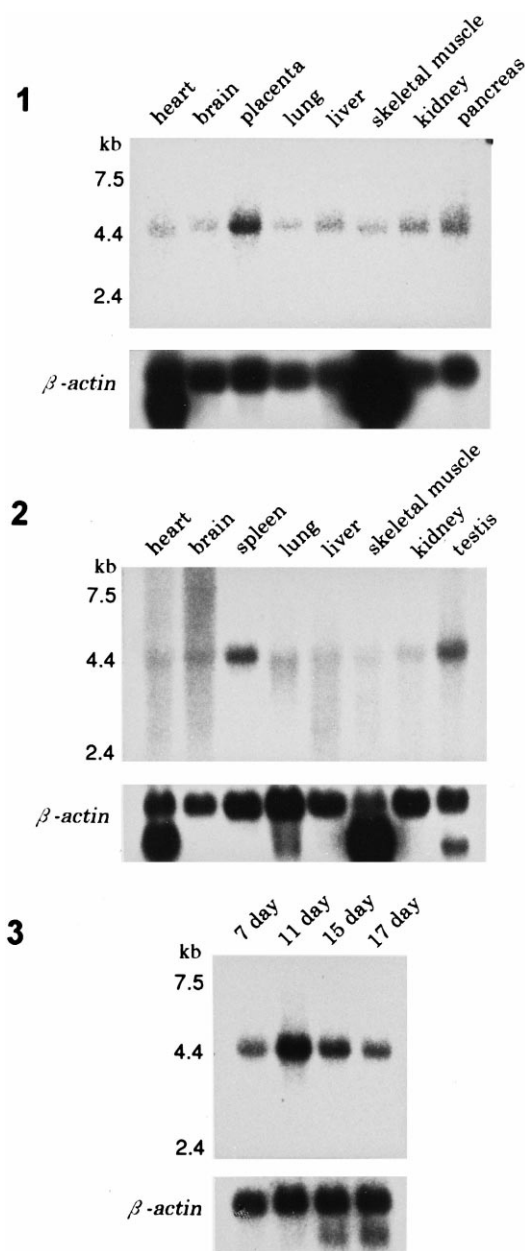


Fig. 3. Expression of hTIM1 and mTim1 analyzed by Northern blot analysis. The filters (1, human; 2, mouse; and 3, mouse embryo) were probed with species-specific *Tim1*, and β -actin.

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,

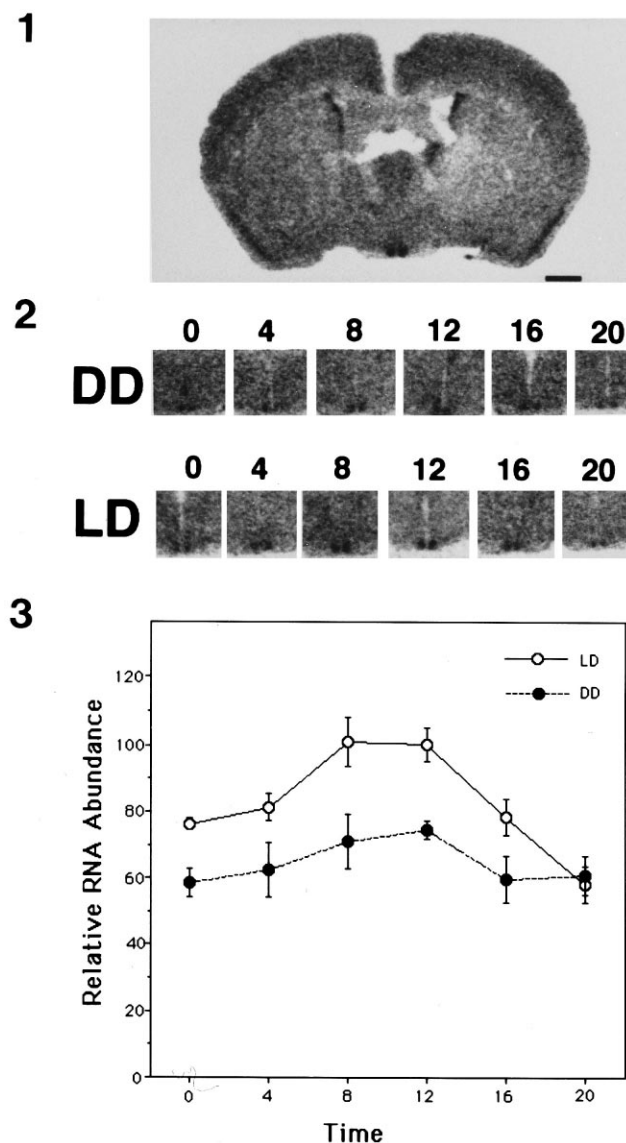


Fig. 4. Expression of hTIM1 and mTim1 analyzed by in situ hybridization. 1: In situ hybridization of mTim1 in the mouse forebrain during the daytime of LD condition (ZT8). Scale bars=1 mm. 2: Representative in situ hybridization autoradiograms showing SCN at ZT0, 4, 8, 12, 16 and 20 in LD and CT0, 4, 8, 12, 16 and 20 in DD. 3: Rhythmic expression of mTim1 in LD (open circle) and DD conditions (closed circle) by quantitative in situ hybridization. Each value is the mean \pm S.E.M. ($n=5$). Plots of RNA cycling set mean peak value to 100.

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, *mTim1* exhibited no clear circadian expression in the mouse SCN (Fig. 4), in contrast to the robust circadian oscillation of *dtim* in the fly brain [6]. This result suggests a difference between the utilization of *mTim1* and *dtim* 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 silkworm brain are different from those in *Drosophila* [29]. Establishment of genetic mutant mice for *mTim1* 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 *mTim1* function in the mammalian clock system.

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