TIMELESS: A link between fly's circadian and photoperiodic clocks?

Jaroslav PAVELKA[1,2*], Kimio SHIMADA[1] and Vladimir KOSTAL[2]

[1]Institute of Low Temperature Science, Hokkaido University, Sapporo, Japan
[2]Institute of Entomology, Academy of Sciences, Branišovská 31, CZ-370 05 České Budějovice, Czech Republic

Abstract. Potential involvement of circadian clock genes in so far unknown mechanism of photoperiodic time measurement is an important question of insect life-cycle regulation science. Here we report about the cloning of full-length cDNA of the structural homologue of the *Drosophila*'s *timeless* gene in *Chymomyza costata*. Its expression was compared in two strains: a wild-type strain, responding to short days by entering larval diapause and a *npd*-mutant strain, showing no photoperiodic response. The *timeless* mRNA transcripts were not detectable by Northern blot analysis in the fly heads of *npd*-mutants, while they were detectable and showed typical daily oscillations in the wild-type strain. After disrupting the normal process of *timeless* transcription in the wild-type strain by injection of *timeless* double-strandRNA into early embryos of wild-type (RNAi method: Kennerdell & Carthew 1998, 2000), a certain proportion of the individuals adopted a *npd*-mutant phenotype, showing no-diapause in response to short-daylength. Cloning of genomic DNA fragments revealed that *npd*-mutants carry a different allele, *timeless[npd]*, with a 13-bp insertion in an intron positioned within the 5'-leader sequence. Genetic linkage analysis showed that the 13-bp insertion (a marker for *timeless[npd]*) and the absence of response to short days (a marker for *npd*-phenotype) are strictly co-inherited in the F2 progeny of the reciprocal crosses between wild-type and *npd*-mutant flies. Such results indicated that the locus *npd* could code for the *timeless* gene in *C. costata* and its product might thus represent a molecular link between circadian and photoperiodic clock systems in this fly.

INTRODUCTION

Circadian clock systems allow organisms to anticipate daily changes in environmental factors and appropriately tune their physiological state. Within the past three decades, starting with identification of the *period* gene locus in *Drosophila melanogaster* (Konopka & Benzer, 1971), enormous progress has been made in describing the molecular details of circadian clocks in cyanobacteria, fungi, plants, insects and mammals (for review see: Dunlap, 1999; Harmer et al., 2001).

The photoperiodic time measurement system (photoperiodic clock) perceives seasonal change of day-length or night-length (for recent reviews on photoperiodism in different organisms see: Samach & Coupland, 2000; Goldman, 2001; Roenneberg & Merrow, 2001; Suzuki & Johnson, 2001; Wehr, 2001), which signals for seasonally cycling deterioration of conditions for life/development and allows organisms to prepare in advance, by entering diapause state in insects, for example (Denlinger, 2000, 2002; Saunders, 2002). Physiological mechanisms of the photoperiodic time measurement system remain obscure. Part of the scientific community accepts that certain elements with circadian oscillatory nature may be functionally involved in photoperiodic clocks. This hypothesis, formulated first by Bünning (1936) for plants, has later received support from other organisms including insects (Saunders, 2002). Nevertheless, the evidence so far gathered is not conclusive enough (Tauber & Kyriacou, 2001; Veerman, 2001).

A central question in the study of physiological basis of photoperiodism is whether the known molecular elements of circadian clocks may serve as functional parts of photoperiodic clock system. In insects, Saunders et al. (1989) analyzed photoperiodic response (diapause induction) in *D. melanogaster* carrying different mutant alleles of the *period* locus. They found that a double deletion of the *period* gene failed to prevent the ability of female flies to discriminate between long and short days. However, genetic dissection of geographical variation in diapause and eclosion rhythmicity in a closely related fly, *D. littoralis*, implied that the same central clock may be used for photoperiodic and circadian time measurements (Lankinen, 1986). More recently, Goto & Denlinger (2002) reported that the amplitude and phase of *period* and *timeless* expression is affected by photoperiod in *Sarcophaga crassipalpis* fly heads. Experiments with mammals showed that some of the elements of central circadian clock (mPer, *tau*) might directly participate in decoding photoperiodic time (Loudon et al., 1998; Majercak et al., 1999; Messager et al., 1999; Nuesslein-Hindelsheim et al., 2000; Sumová et al., 2002).

In the light of above information, we started an effort to elucidate the potential role of circadian clock genes in photoperiodism of the fly, *Chymomyza costata* (Diptera: Drosophilidae). The mature larvae of the wild-type (Sapporo) strain of the fly enter diapause in response to subcritically short days or low temperature (Riihimaa & Kimura, 1989; Kostal et al., 2000a). In 1983, Riihimaa & Kimura (1988) succeeded in selecting a mutant strain of flies which did not respond to a photoperiodic signal but were able to enter diapause at 11°C; the authors named the strain as NPD (Non-Photoperiodic-Diapause). Later,

```
C.cos                                    MDWLLATPQLHSAFSSLGALVGDTYVVSPDALAILEE    37
                                         :::::::::::::::. :::::::: .::::: ::::::::
D.mel    MSRVRQLHNHIWNNQNFDKVKSVMDWLLATPQLYSAFSSLGCLEGDTYVVNPNALAILEE    60


C.cos    INYKLIYEDQTLRTFRRAIAFGQNVRSDLIPVLENTKDDAVLESVIRLLVNLTVPVECLF    97
         :::::. :::::::::::::.:::::::::::.:::.:::::::::::. ::::::::::::
D.mel    INYKLTYEDQTLRTFRRAIGFGQNVRSDLIPLLENAKDDAVLESVIRILVNLTVPVECLF   120


C.cos    SVDVMYRTEMGRHTIFELNKLLYNSKEAFTDPKSTKSVVEYMKHILDSETKLSPQKCDQI   157
         :::::::::.:::::::::::::: ::::::. ::::::::::::::::.:.:::::.::::
D.mel    SVDVMYRTDVGRHTIFELNKLLYTSKEAFTEARSTKSVVEYMKHILESDPKLSPHKCDQI   180


C.cos    NNCLLLLRNILHIPETNANFIKPMLQSSSRHGTSMQNTILWNLFIQSIDKMLLYLMTCPQ   217
         ::::::::::::::::: :.  :::.:.:  :: :::::::::::::::::.:::::::::::
D.mel    NNCLLLLRNILHIPETHAHCVMPMMQSMP-HGISMQNTILWNLFIQSIDKLLLYLMTCPQ   239
                                    -----32 aa region---------------
C.cos    RALWGVTMVQLIALIYKDQHISTLQKLLNLWFEASLSESSEDNESNTTPPKQGSGDYSPM   277
         ::.:::::::::::::::::::.::::::.:::::::::::::::::::.:::::::::.:::
D.mel    RAFWGVTMVQLIALIYKDQHVSTLQKLLSLWFEASLSESSEDNESNTSPPKQGSGDSSPM   299


C.cos    LTSDPTSDCSEKDGDERRQT--LREGTDATLQEVSRKGQEYQDAMFGVPAKKLECAETSS   335
         ::::::::: :.: ::    :  .:.: ::::::::::::::::::.:  .::: .:  ..:
D.mel    LTSDPTSDSSDNGSNGRGMGGGMREGTAATLQEVSRKGQEYQNAMARVPADKPDGSEEAS   359
                              -------acidic domain----------
C.cos    EVT---SEAPSSPQQ----------------HWKVTEPPEPEKEMAMAAITEQ----QA   371
         ..:   :: ::::: ..             . : .::: .::::::.: : .:::    .:
D.mel    DMTGNDSEQPGSPEQSQPAGESMDDGDYEDQRHRQLNEHGEEDEDEDEVEEEEYLQLGPA   419


C.cos    SMIMQLTQ-PADNAKTTTNTTCTANQ-----ERFKQPPPLPVRPLTCTQAEKLGEMADET   425
         : ::  ::  :::: .:::.: ::. .     :: ::::::::::::: :. .. :...::
D.mel    SEPLNLTQQPADKVNNTTNPTSSAPQGCLGNEPFKPPPPLPVRASTSAHAQ-MQKFNESS   478
                                                              ---
C.cos    YPKHVSAVKRGQKSPHSGQLHLTKGKCFPQKRECASSQSELSDCGYGTQVENQESISTSS   485
         : .::::::..::::: :::: ::::: ::::::::.:::::::::::TQVENQESISTSS
D.mel    YASHVSAVKLGQKSPHAGQLQLTKGKCCPQKRECPSSQSELSDCGYGTQVENQESISTSS   538
                                                        Δ tim^SL
                 --------------PID1-------------------====NLS====-----PID1------
C.cos    NDDDAPQGKPQHQKPPCNTKPRNKQRNIVTPLDKKEQRRKKLVKRSKSNLINMKGLCQHT   545
         ::::.::::::::::::::::::::.::.:..: :::::.::::::::::.:::::::.:::
D.mel    NDDDGPQGKPQHQKPPCNTKPRNKPRTIMSPMDKKELRRKKLVKRSKSSLINMKGLVQHT   598
         ------------
C.cos    PTDDDISNLLKEFTVDFLLKGYNYLVKVLHLHITSNAKVSIDTSHFFWLVAYFLKFAAQL   605
         :::::::::::KEFTVDFLLKGY..::. :.:.::::::.:::::::::::.:::::::::
D.mel    PTDDDISNLLKEFTVDFLLKGYSYLVEELHMQLLSNAKVPIDTSHFFWLVTYFLKFAAQL   658
                  Δ tim^L1
C.cos    ELDMEHIDTILNFDVLTYLTSEGVSLCEQLELNARQEGSDLKPYLRRMHLVVTAIREFLQ   665
         ::::::::::: .:::.:::.:::::::::::::::::::::::::::::::::::::::::
D.mel    ELDMEHIDTILTYDVLSYLTYEGVSLCEQLELNARQEGSDLKPYLRRMHLVVTAIREFLQ   718
                                            --------------------------
C.cos    AIETYSKVTHLSEDDRTQLLKPQIQISAATDLRSLFVLLLRRFNPSIHSKQYLQDLVVTN   725
         ::.:..::::.::.::.:::.::::::.:.:::::::::::::::::::::::::::::::
D.mel    AIDTYNKVTHLNEDDKAHLRQLQLQISEMSDLRCLFVLLLRRFNPSIHSKQYLQDLVVTN   778
                                  -------------------------PID2---------------------
C.cos    HKLMLVLDRAAKLEGGQSVYLTEHLLQFATPEVMHYYGILLEDFNYNGEFLNDCIFTMMH   785
         : ::.:::..:::::.:: .: ::.::::::.:::::::::::DFN.NGEF.NDCIFTMMH
D.mel    HILLLILDSSAKLGGCQTIRLSEHITQFATLEVMHYYGILLEDFNNNGEFVNDCIFTMMH   838
                                                         Δ tim^L2
         --------------------------------------------------------
C.cos    HIGGDLKQIGVLFQPIILKTFSRLWETDNGLCDDWTDLIAYVIRKIMSTHPVS-VDLRTS   844
         :::::::.::::::::::::::.::.:: : .:::::.:::.:::..:..::::  .::..
D.mel    HIGGDLGQIGVLFQPIILKTYSRIWEADYELCDDWSDLIEYVIHKFMNTPPKSPLTIPTT   898
         --------------------------------------------------
C.cos    SLTEMTKEHNLQNNLFTWTQEDMDTLYWYYVQSKKSNDVVGKIVKLFSNNGNKLKSRISI   904
         ::::::::::.:.::: .: ::::::::::::::::.:::.:::::::::::::::.::::
D.mel    SLTEMTKEHNQEHTVCSWSQEEMDTLYWYYVQSKKNNDIVGKIVKLFSNNGNKLKTRISI   958


C.cos    IQQLLAQDMINLLEYDDLMKFEEAEYQRSLLNTPTSGTNASGLE-----SGKPSNDTQML   959
         :::::.:: : ::::::::::::.:::::.:: ::::  ..::.     ::::::::.::::
D.mel    IQQLLQQDIITLLEYDDLMKFEDAEYQRTLLTTPTSATTESGIEIKECAYGKPSDDVQIL  1018


C.cos    LDLIIKDNKQQHLIWLQQLLLECCYIKLKLRSGNSCST-DAIMEPVAYHCILMQKSIPIV  1018
         :::::::.:: ::::::.::::::::.:::::::::.   : ::::::::::: ::::::::
D.mel    LDLIIKENKAQHLLWLQRILIECCFVKLTLRSGLKVPEGDHIMEPVAYHCICKQKSIPVV  1078


C.cos    QWNNDQSAIMLYQPFVLLLHKLGIQLPADAGTIFARIPDYWTPETMYGVAKKLGTLDKLT  1078
         ::::.:: .:::::::::::::::::::::::.:::::PDYWTPETMYG.AKKLG.LDKL.
D.mel    QWNNEQSTTMLYQPFVLLLHKLGIQLPADAGSIFARIPDYWTPETMYGLAKKLGPLDKLN  1138
                                            Δ tim^rit
C.cos    LKFDPIELEVASSTTPR--HCMKSLNSLSSVSSLDVDMPELEELPLIPEVDAAIEKAH--  1134
         ::::  ::: ::::::    ::: ::::::::::::::::.: ::: ::::::::.:::::
D.mel    LKFDASELEDATASSPSRYHHTGPRNSLSSVSSLDVDLGDTEELALIPEVDAAVEKAHAM  1198


C.cos    -------EIFVAPKAKIVIQLFRYYAGCYTSVPNWLQLVMRSKCIQQSVPSRPATDISDC  1187
                ::::: :: :.  .: :.. :: :.:::PNWLQLVMRSKC..:..:  ... .:::
D.mel    ASTPSPSEIFAVPKTKHCNSIIRYTPDPTPPVPNWLQLVMRSKCNHRTGPS---GDPSDC  1255
                                  ----------------------CLD-----------------------------
C.cos    TGA-----DDESMHSSSQQAPTQQLLATSNSSNVNHATTLGCQ--PSIIGAHNDNSSSSG  1240
         .:.     :: :: ::.::: :. :  :.:.  :.. ::  :.   .:.:.:::..::::
D.mel    VGSSSTTVDDEGFGKSISAATSQA--ASTSMSTVNPTTTLSLNMLNTFMGSHNENSSSSG  1313
         -------------------------------------------------------------
C.cos    CGGTDSSQSMAAVVSNTRTTGNNTPRKTAEAISRKTTHMYEDAAMKS-FQRLQATNSHFS  1299
         :::: ::.:::.:.::::  :::         :..:: :::.:..:: F.RL..:::::::
D.mel    CGGTVSSLSMVALMSTGAAGGGG---------NTSGLEMDVDASMKSSFERLEVNGSHFS  1364
         ----------
C.cos    RANNLEQQYSALVASIYENEMA-NCDNISMASDLEHMYVSDEEDTHENHEQRVPYYH     1355
         ::::::.:::: ::.:::..:.   :.:.:.:SDL..MYVSDE.D.:: .: ::::::
D.mel    RANNLDQEYSAMVASVYEKEKELNSDNVSLASDLTRMYVSDEDDRLERTEIRVPHYH     1421
```

256

the rhythmicity of adult eclosion was studied and genetic dissection experiments confirmed that the non-photoperiodism and eclosion arrhythmicity in the NPD strain were caused by mutation in a single autosomal gene locus *npd* (Riihimaa & Kimura, 1989; Riihimaa, 1996; Lankinen & Riihimaa, 1992, 1997). Formal analysis of the photoperiodic clock's function in *C. costata* revealed that an element with a circadian-oscillatory nature participates in photoperiodic time measurement (Yoshida & Kimura, 1995; Kostal et al., 2000b). Shimada (1999) sequenced the *per* homologue in *C. costata* and found that the NPD flies have a 6-bp deletion site (deleting two amino acids) in the coding region, in addition to a few other point mutations (see accessions Nos. AB014476 and AB014477 in DDBJ/EMBL/GenBank database). These mutations were found to be primarily neither responsible for the loss of the eclosion rhythm nor for the loss of photoperiodic response in NPD strain. Nevertheless, the daily and circadian oscillations of the *per* gene mRNA abundance, that were detected in the Sapporo strain, were lost in the NPD strain, which suggested an impaired function of the central circadian clocks in *npd*-mutants (Kostal & Shimada, 2001). The gene *timeless* codes for a protein (TIM), which forms a heterodimer with PER within the cytoplasm of *Drosophila* clock neurons; together they translocate to the nucleus where they repress their own transcription with the help of two dimerizing transcription factors, CYCLE and CLOCK, binding to E-box sequences in the *period* and *timeless* promoters. Such a negative feedback loop forms the core of the central circadian clock in *Drosophila* (Sehgal et al., 1994; Allada et al., 1998; Darlington et al., 1998; Rutilla et al., 1998).

Here we report an introductory analysis of the structure and expression of the gene *timeless* in two strains of *C. costata* (Sapporo and NPD). We found that the mRNA transcripts of *C. costata*'s structural homologue of *Drosophila*'s *timeless* gene are not detectable by Northern blot analysis in the NPD strain fly heads, while they are detectable and show typical daily oscillation in the Sapporo strain. After disrupting the normal process of *timeless* transcription in the Sapporo strain by the mean of RNA interference methodology (RNAi, Kennerdell & Carthew 1998, 2000), a certain proportion of the individuals that were treated with *timeless* dsRNA adopted a *npd*-mutant phenotype, showing no-diapause in response to short daylength. Comparing the sequences of the genomic DNA fragment between the two strains revealed that the *npd*-mutants carry a different allele, *timeless^{npd}*, with numerous point substitutions and a 13-bp insertion in an intron positioned within the 5'-leader sequence of the mRNA. Genetic linkage analysis showed that the 13-bp insertion (a marker for *timeless^{npd}*) and the absence of response to short days (a marker for *npd*-phenotype)

are strictly co-inherited in the F2 progeny of the reciprocal crosses between Sapporo and NPD flies. Such results indicate that the gene *timeless* might play a direct role in photoperiodic induction of diapause in *C. costata* and its product might thus represent a molecular link between the circadian and photoperiodic clock systems.

MATERIALS AND METHODS

Insects

Two strains of *Chymomyza costata* were used for experiments: a wild-type (Sapporo) strain originally collected in Sapporo (43°N), Japan in 1983; a *non-photoperiodic-diapause* (*npd*)-mutant strain (NPD) which was isolated by Riihimaa & Kimura (1988) from wild-type flies collected in Tomakomai (42.3°N), Japan. Larvae, pupae and adults were cultured on an artificial diet of Lakovaara (1969) under a constant temperature of 18°C and a short-day (SD) photoperiod (10h light : 14h darkness) that induces larval diapause in 100% of the population of Sapporo strain but no-diapause in the NPD strain.

DNA, RNA extractions

Genomic DNA was extracted by homogenization of whole adults in DNAzol Reagent (GibcoBRL) using the manufacturer's instructions. Precipitated DNA was dissolved in water.

For total RNA extraction, adults were frozen on a metal block submerged in liquid nitrogen. Their heads were separated, RNA extracted by homogenization in ISOGEN (Nippon Gene, Japan) using the manufacturer's instructions, and dissolved in DEPC-treated water. Traces of DNA in total RNA samples were digested with DNase I (Roche), purified total RNA was isolated using standard phenol/chloroform extraction and re-dissolved in DEPC-treated water. Total RNA was extracted also from whole white puparia (freshly pupariated larvae, before formation of pupa), either from non-injected controls or from the "positive" individuals injected with *timeless* dsRNA in the RNAi experiment (see below).

Cloning of *timeless* and *β-tubulin* homologues, preparation of probes for Northern blot analysis

The *timeless* and *β-tubulin* cDNA fragments were obtained using the standard RT-PCR method. In the first step, cDNA synthesis was performed with ThermoScript RT-PCR System (GibcoBRL) according to the manufacturer's instructions. Total RNA (2mg) was primed with 2.5μM oligo (dT)$_{20}$ and reverse transcribed using 15U of the ThermoScript enzyme in 20μl reaction mixture. In the second step, aliquots of cDNA (0.5μl) were amplified by a standard PCR with 2.5U *Taq* DNA polymerase (Takara, Japan) in 50μl reaction mixture (10mM Tris-HCl, 50mM KCl, 1.5mM MgCl$_2$, 0.8mM dNTP, pH 8.3) under the following thermal conditions: 95°C, 3 min; 30 cycles of 94°C, 30 sec; 55°C, 30 sec; 72°C, 2 min. The annealing temperature was changed between 50 and 65°C depending on T$_m$ values of primers. The time at 72°C was extended to 3 or 5 min depending on the length of target cDNA. PCR primers tim11, tim12 and tub1, tub2 (Table 1) were designed on the basis of published *timeless* and *tubulin* cDNA sequences from other drosophilid species. The PCR products were digested with *Eco*R I or *Sau*3A I restriction enzymes, and cloned into pBluescript SK(+) vectors (Stratagene).

Fig. 1. Alignment of TIMELESS amino acid sequences from *Chymomyza costata* (C.cos) (EMBL/GeneBank/DDBJ accession number, AB073724) and *Drosophila melanogaster* (D.mel) (Swiss-Prot accession number, P49021) (alignment was performed using FASTA software; http://www.genome.ad.jp). Several known functional motifs are shown using dashed line above the sequence and the sites to which the *tim^{SL}*, *tim^{L1}*, *tim^{L2}* and *tim^{rit}* mutations have been mapped are indicated by triangles (see text for explanations). Identical and positive amino acids are indicated by double dot (:) and single dot (.) marks, respectively.
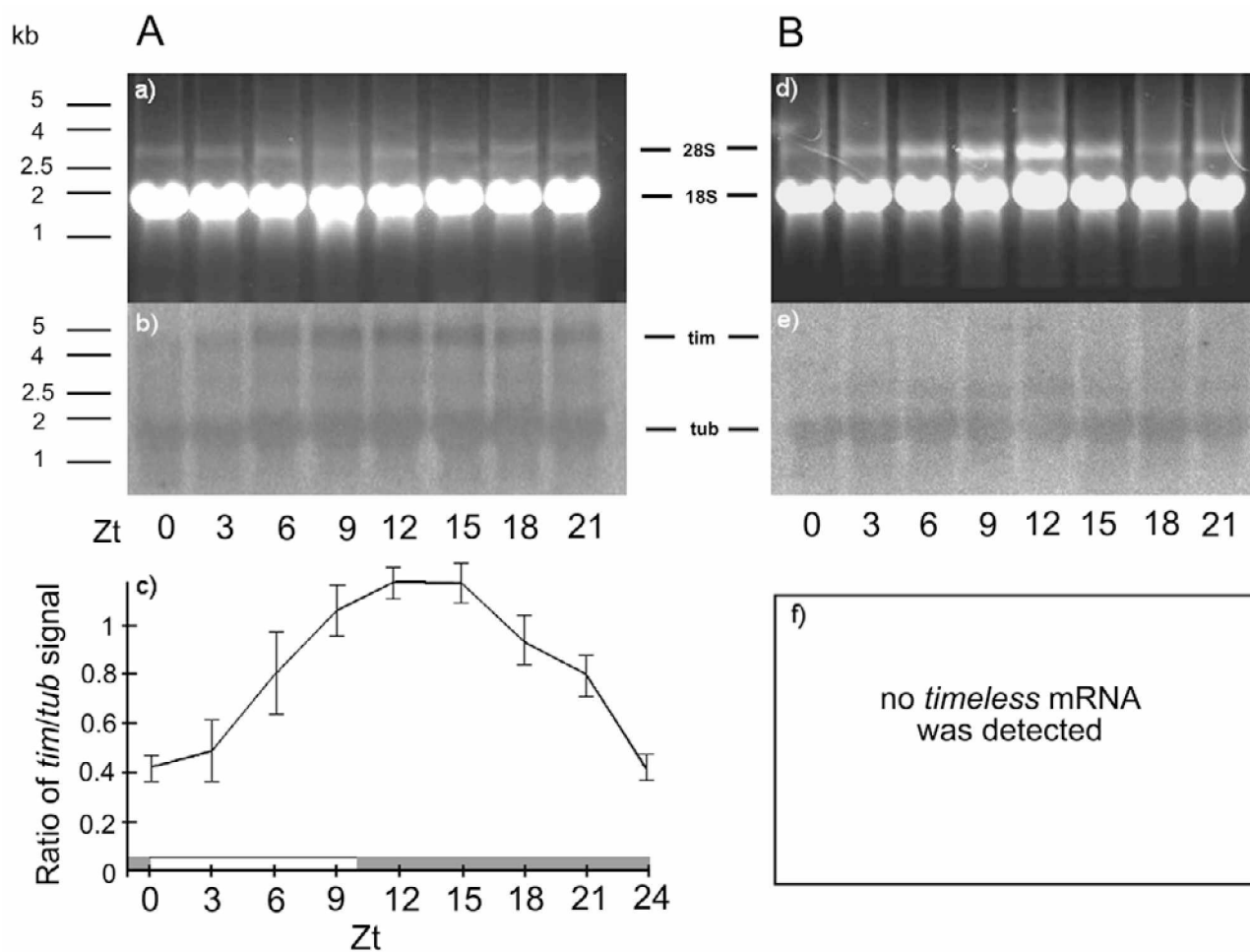
Fig. 2. Northern hybridization analysis of the expression of *timeless* gene in adult heads of *Chymomyza costata* of the (A) wild-type (Sapporo) and (B) *npd*-mutant (NPD) strains. Samples of total RNA were taken at 3h-intervals of Zeitgeber time (Zt) (white and dark portions of the *x*-axis represent light and dark phases, respectively). Total RNA was loaded on formaldehyde-agarose gel (20μg per lane), electrophoresed at 100V for 90 min and stained by ethidium bromide (part a). After transfer to nylon membranes, RNA was hybridized sequentially with ^{32}P-labeled probes for *timeless* (tim) and ß-*tubulin* (tub) (part b). The quantified radioactive signal of *timeless* hybridization (normalized to signal of ß-*tubulin*) is plotted in part c) of the Figure. Parts a) and b) depict one of three replications of the experiment; part c) shows mean ± S.E. results of three replications.

The *timeless* and ß-*tubulin* cDNA clones were used as sources for probes for Northern blot analysis. The *timeless* probe, isolated from the clone by EcoR I digestion, had 321 nucleotides. The deduced sequence of 107 amino acids corresponded to amino acids 610–716 of *D. melanogaster* TIMELESS protein and 87% of amino acids were identical between the two insect species. The ß-*tubulin* probe was isolated from the clone by digestion using *Pst* I and *Xba* I restriction enzymes. It consisted of 665 nucleotides. The deduced sequence of 221 amino acids corresponded to amino acids 84–304 of *D. melanogaster* ß-TUBULIN protein and 90% of amino acids were identical between the two insect species.

Sequencing the full-length *timeless* cDNA

Two *timeless*-gene-specific primers tim15, tim16 (Table 1) were designed based on cDNA sequence of the cloned *timeless* fragment. The upstream and downstream coding regions were amplified by RT-PCR with two sets of primers: upstream: tim16, tim21; downstream: tim15, tim28 (Table 1). 5'- and 3'- unknown sequences flanking to the coding region were amplified by 5' Rapid Amplification of cDNA Ends (5'RACE) and 3'RACE Systems, respectively, according to the manufacturer's

instructions (GibcoBRL). Gene-specific primers tim31, tim35 (Table 1) were used to prime the target sequences. RT-PCR products, digested with or without EcoR I, were cloned into pBluescript SK(+) (Stratagene) or pGEM-T Easy (Promega) vectors. The clones carrying target genes were cultured in Terrific Broth. The plasmid DNA was extracted by the standard alkali lysis and purified by precipitation with polyethylene glycol (Sambrook et al., 1989).

Purified plasmid DNA was sequenced by using THERMO Sequenase II dye terminator cycle sequencing kit (Amersham Pharmacia Biotech). Cycle sequencing reactions were carried out according to the manufacturer's instructions. The precipitated products were re-dissolved in sample loading buffer (Amersham Pharmacia Biotech) and were analyzed on ABI PRISM 377 DNA Sequencer (Applied Biosystems).

Northern blot analysis

Adult flies of the Sapporo and NPD strains maintained at 18°C/SD for 7 days (since adult emergence) were subjected to Northern blot analysis. The fly heads (100/sample) were collected at 3-hour intervals of Zeitgeber time throughout the photoperiodic cycle. Total RNA was extracted by the standard
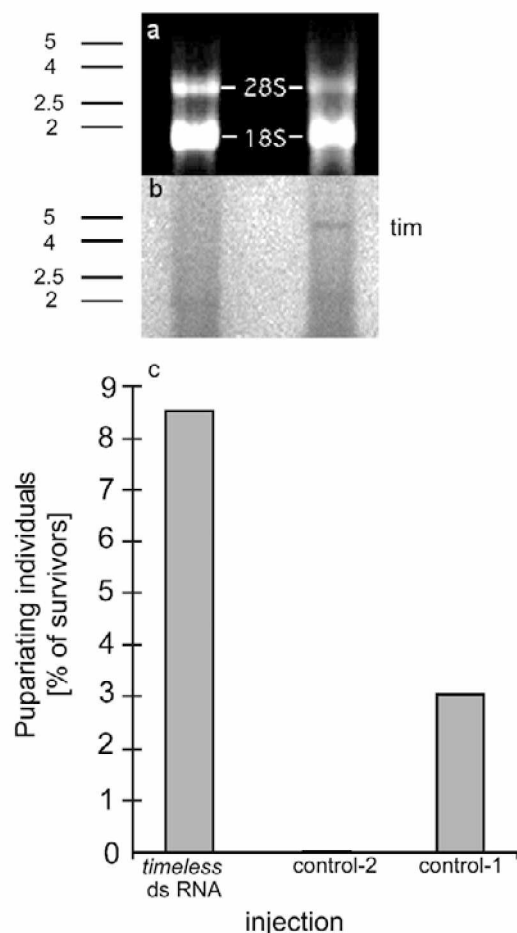
Fig. 3. RNA interference. Total RNA was extracted from 15 pupariating individuals of *Chymomyza costata* (wild-type, Sapporo strain) that were differently treated in early embryonal stage (from the left): injected with the *timeless* dsRNA dissolved in injection buffer under halocarbon oil; treated with halocarbon oil without any injection (control-2); injected with injection buffer alone under halocarbon oil (control-1). The upper part (a) shows total RNA (5.5μg in each lane) separated on formaldehyde-agarose gel and stained by ethidium bromide. The middle part (b) shows Northern hybridization with ^{32}P-labeled *timeless* probe on a nylon membrane. The lower part (c) show proportions of pupariating individuals in each treatment (total numbers of survivors were: 504 after dsRNA injection; 258 in control-2; 82 in control-1).

protocol described above. Each aliquot (20μg) was separated by formaldehyde-agarose gel electrophoresis, transferred to nylon membranes (Hybond-N⁺, Amersham) and hybridized with radioactive ^{32}P-labeled probe for *timeless*. After washing out the *timeless* probe, the separated RNA on nylon membranes was hybridized with *β-tubulin* ^{32}P-labeled probe. The membranes were exposed to a BAS-1500 Imaging Plate (Fujifilm, Japan) for 2 hours and quantitatively analyzed using the NIH Image software (http://rsb.info.nih.gov/nih-image/).

RNA interference

The RNAi method (Kennerdell & Carthew, 1998, 2000) was applied in an attempt to silence the *timeless* gene expression in the Sapporo strain larvae and observe its effect on photoperiodic phenotype (developmental response to short days). Template

TABLE 1. Primer sequences.

Purpose name	Sequence (5' → 3')
Cloning	
tim11	AAAGAATTCTGGGGNGTNACNATGGT
tim12	AAAGAATTCCDATRTGRTGCATCAT
tub1	AAGAGCTCTGGAGCCMGGYACSATGGACTCKGT
tub2	AAGAGCTCGTCTTCACGTTGTTGGGGATCCA
Sequencing cDNA	
tim15	GCTCAAGGGCTATAACTATTTGG
tim16	ACCACAAGATGCATGCGACGC
tim21	AAGAATTCATGGAYTGGTTRCTNGCNAC
tim28	AAGAATTCRTCYTCRTCRCTNACRTACAT
RACE	
tim31	GCTTAAAGCGCTCTTGGTTAGCTGTGC
tim35	AAGAATTCGGTGGCATCTATATACGAAAA
Sequencing intron	
tim23	AAGAATTCACCCAATGCATYGGGGCTGACCACAT-AGGT
int1	AAGAATTCNNNAGGGTAAG
tim26	AAGAATTCAAGCTGCTAGTCGCCATAGCAG
tim27	AAGAATTCGTGTTATCATCCTGCCACG
Genetic linkage analysis	
tim24	CGACACGTTGAACGCTCATACG
tim29	TTCAGAGCGTGATTTTGCAGCAGTC

DNA for synthesis of double-stranded (ds) RNA was produced by a standard PCR using primers tim16, tim21 (Table 1) with the T3 and T7 promoters added to primer sequences [reaction conditions: 5U *Taq* DNA polymerase (Takara, Japan) in 200 μl reaction mixture (10mM Tris-HCl, 50mM KCl, 1.5 mM MgCl₂, 0.8mM dNTP, pH 8.3); thermal conditions: 95°C, 3 min; 30 cycles of 94°C, 30 sec, 60°C, 30 sec, 72°C, 5 min]. Single strand *timeless* RNA was transcribed using the T3,T7 RNA polymerase on the genomic DNA template according to the instruction manual (Stratagene). Remaining DNA was digested by DNAase (Roche). Single strands of RNA with T3 and T7 ends were mixed in injection buffer [IB, 5mM KCl, 0.1mM NaH₂PO₄, pH 6.8 (Rubin & Spradling, 1982)], heated at 100°C for 1 min, and kept at room temperature for 18h. Aliquots were frozen at −80°C. Resulting dsRNA had 1966 nucleotides, the deduced sequence of 655 amino acid corresponded to amino acids 24–711 of *D. melanogaster* TIMELESS protein (76% amino acids were identical between the two insect species). The dsRNA was dissolved in IB and approximately 0.2 fmol (ca 0.3 μl of IB) aliquots of dsRNA were injected, under halocarbon oil (Sigma), into the ventral side of the posterior domain of *C. costata* embryos, Sapporo strain. The embryos were not more than 1h old. Control embryos were either injected with injection buffer alone (control-1) or were only exposed to halocarbon oil (control-2). After the treatment, the embryos and larvae were reared under short-day conditions, which promote entrance into diapause (no-pupariation) in untreated Sapporo strain larvae. Those dsRNA-treated individuals that pupariated ("positive" individuals) were collected in the stage of white puparium and total RNA was extracted for the analysis of the presence of *timeless* mRNA transcripts by Northern (15 puparia were pooled for one sample, see above for RNA isolation).

Sequencing the intron

Sequential Thermal Asymmetric Interlaced (TAIL)-PCR (Liu et al., 1995) was carried out to determine genomic sequence flanking to the *timeless* coding region. The genomic DNA from the wild-type *C. costata* was amplified in a 50μl PCR buffer [(500ng DNA, 0.8mM dNTP, 2.5U of *Taq* DNA polymerase (Takara, Japan)] using 0.2μM of the *timeless*-specific antisense primer tim23 and 1mM of a degenerate sense primer int1 (Table 1). After 3min of denaturation at 95°C, the DNA fragments were amplified for 5 cycles of 60 sec at 94°C, 60 sec at 65°C,

```
tim Sap -1399:GTGTTATCATCCTGCCACGTTACGTAGCTGCACATTGCATTGACTGTGGCTGTCCGTCTC
tim NPD -1421:GTGTTATCATCCTGCCACGTTACGTAGCTGCACATTGCATTGACTGTGGCTCTCCGTCTC
              ************************************************** ********

tim Sap     :CATATAACTGCTTGAGTGACAGTTACTGCAGTTAACATGCTACTTGTACGTGGTTACATG
tim NPD     :CATATAACTGATTGAGTGACAGTTACTGCAGTTAACATGCTACTTGTACATGGTTACATG
              ********** ************************************** **********

tim Sap     :CATTCTTGAGATTTCTTTGACATATAGTACTGTGAAAAGGTGGTGGCCAGCACATAGGTA
tim NPD     :CATTCTTGAGATTTCTTTGACATATAGTACTGTAGAATGGTGGTGGCCAGCACTTACGTA
              ********************************* **  ** ************  ** ***
                             tim24
tim Sap     :GCTATTTTTATATGCGACACGTTGAACGCTTATACGTTGTTTGGGATTCACAATGAATGA
tim NPD     :GCTATTTTTATATGCGACACGTTAAACGCTCATACGTTGTTTGGGATTCACAATGAATGA
              ***********************.**** ***** *************************

tim Sap     :ACACTGCAAAGGTTTTTTGTACTATGGGTGGTGGGG------------TGCTGCTATGG
tim NPD     :ACATTGCAAAGGTTTTTTGTACTATGGGTGGTGGGGTACGAAAATTTAATGCTGCTATGG
              *** ***********************************          **********

tim Sap     :CGACTAGCAGCTTATGAAAGGCAAAGGCATTTTTTTATGATGATTTTTGAATCGAAAATT
tim NPD     :CGACTAGCAGCTTATGAAAGGCAAATATTTCTCTTTATTTTTATGATGGTTTTTGAATGG
              ************************    * * *****  * **  *  *  *    **
                                                  tim29
tim Sap     :AGAAATTTATATAAATGCTTTTTGTTTAAAATAAAAACGCGACTGCTGCAAAATCACGCT
tim NPD     :AAAAGTTTATATAAACGCTTTTTGTTAAAAATAAAAACGTGACTGCTGCAAAATCACGCT
              * ** ********** *********** **************.*****************

tim Sap     :CTGAAAAATATTGAACCAAGAAATATTCTCATCTGAATTAATTATGCAAAAACTACGCAA
tim NPD     :CTGAAAAGTATTGAACAAAAAAATATTCTCATTTGAATTAATTATGCACAAACTACGCAA
              ****** ******** **  ************ ************** ***********

tim Sap     :TATTCATCATCTATTAAATAAACATTGGCTATATATTTTAATGTATTTAGTACATATTCA
tim NPD     :TATTCATCATCTATTAAATAAAGATTGGCTATATATTTTAATGTATTTGGTACATATTTA
              **********************.******************** ******* ******* *

tim Sap     :CAGAGTTAGCCGTCGTTATTGCCAACTGAGTTAATATTCGATATTTTTTTAGTCCCTCTA
tim NPD     :AAGAGGTAGCCGTCGTTATTGCCAACTGAGTTATTATTCGATATTTTTTTAGTTCCTCTA
              ***** *************************** ******************* ******

tim Sap     :GAGTTTGCCATATGCAATTTTTTGTTACTTTATAATGCCTATAACTAGTGGACGACCAAG
tim NPD     :GAGTTTGCAATATGCAATTTTTTGTTACTTTATAATGCCTATAACTTGTGGACGACCAAA
              ******** ************************************* **********

tim Sap     :TCGCTTCAAACGATACCTCATTTGTCGAATTATGATATACAGCTTAGAAGTTACGAGGGA
tim NPD     :ACGCTTCCAACGATACCTCATTTGTCAAATTATGATATACAGTTTAGAAGTTAAGAGGGA
              *********************** **********.******* **********  ******

tim Sap     :ACAGATG--CA----CTTCTTTGTTGCATTAACTTCTAGTGGAGCATAGGGCCTTCCGAA
tim NPD     :ACAGATGCACATCTTCTTCTTTGTTGCATTAACGCCTAGTAGAGCATAGGGCCTTCCGAA
              ******* **  ***************** ***** ****************

tim Sap     :GATAAACATAAATACAGACATTTATGTCAAAAACAAAACACTCCTTTTTATTCGCCGTAG
tim NPD     :GATAAACACAAATACAGACATTTATGTCAAAAACAAAACACTCCTTTTTGTTCGCCGTAG
              ********.*****************************************.**********

tim Sap     :CCGTGGCTAATTTCTTTTAAAAAGCACTGCTCATCTTCAAGACTTACTCATTTAAGAGTT
tim NPD     :TCGTGGCTAATTTCTTTTAAAAAGCACTGCCCATCTTCAAGACTAACTCATTTAAGAGTT
              .*****************************.*************.************

tim Sap     :AAAAAGCTTTCTTATTAAAAATTCAATAGAATTTTACGAGCAGAAGAC---TGTATTAAA
tim NPD     :AAAAAGCTTTCTTATTAAAAATTCAATAGAATTTTACGAGCAGAAGACTATTGTATTAAA
              ***********************************************.  *********

tim Sap     :GCTAGTGTTCCAAATCTATTGGTTGGTTAGGATAGAACCAAGGCCCGTTCAGCTTATTTT
tim NPD     :GCTAGTGTTCCAAATCTATTGGTTGGTTAGGATAGAACCAAGGCCCGTTCAGCTTATTCT
              **********************************************************.

tim Sap     :GTTGTCGGAGTTGTCTTCAAATTGCGACACTTCTTTTTATGTCTCAAACCAATTTTTTTT
tim NPD     :GTTGTCGCAGTTGTCTTCAAATTGCGACACTTCTTTTTATGTCTCAAACCAATTTTTTTT
              *******.****************************************************

tim Sap     :AAGTTTTAGAATGCACATCAAAAATGTAAAGAATTCTTCTACTTAAAAAGATTATCTCCA
tim NPD     :AAGTTTTAGAATGCACATCAAAAATGTAAAGAATTCTTCTACTTAAAAAGATTATCTCCA
              ************************************************************
                                                  ---- 5'-leader sequence ----
tim Sap     :TGTTGGAAAATGCTAATTTCTTTGTAAAGCTTATTGAAATATAAAATATTCCGTTTAAGT
tim NPD     :TGTTGGAAAACGCAAATTTCTTTTTAAAGCTTATTGAAATATAAAATATTTCGTTTGAGT
              **********.**.*******.******************************.****.***
tim Sap     :AAACTGCATTTAAAATAATATTATCTCATCCAGTTGCAGCCCCAGCCTGCTTACAAA-GG
tim NPD     :AAACTGCATTTAAAATAATATTATCTCTTCCAGTTGCAGCCCCAGCCAGCTTACAAAGGG
              ***************************.*******************.********* **
              ------------------------------------------------------------
tim Sap     :ACCTGTAGCTGGCCTAAACCCTATTAACTAGGGTTGCGTTTCATGCTCTACGCTGATCAT
tim NPD     :ACCTGTAGCTGGCCTAAACCCTATTAACTAGGGTTGCGTTTCATGCTCTACGCTGATCAT
              ************************************************************
              ------------------------------------------------------------
tim Sap     :GTGCTGCAAGTGGCTACAATAATAAATTGTGTCCAAAATCAACGTTATATACAAATC
tim NPD     :GTGCTGCAAGTGGCTACAAATAATAAATTGTGTCCAAAATATCAACGTTATATACAAATC
              ***************************.********************************
              -----------------------------------------+1=================
tim Sap     :CAAGTATTATCATACCAGAAACATCACATAAGCGCCGGGCTATGGACTGGTTACTCGCAA
tim NPD     :CAAGTATTATCATACCAGAAACATCACATAAGCGCCGGGCTATGGACTGGTTACTCGCAA
              ************************************************************
                                                          M  D  W  L  L  A
              ================== coding sequence ==========================
tim Sap     :CGCCACAGTTGCATAGCGCCTTCTCATCATTGGGGGCGCTTGTGGGTGACACCTATGTGG
tim NPD     :CGCCACAATTGCATAGCGCCTTCTCATCATTGGGCGCGCTTGTGGGTGACACCTATGTGG
              *******.**************************  .********************
               T  P  Q  L  H  S  A  F  S  S  L  G  A  L  V  G  D  T  Y  V
              ====================
tim Sap     :TCAGCCCCAATGCATTGGGT  +99
tim NPD     :TCAGCCCCAATGCATTGGGT  +99
              ********************
               V  S  P  N  A  L  G
```

260

and 3min at 72°C followed by 15 cycles of 30 sec at 94°C, 60 sec at 68°C, 3min at 72°C, 30 sec at 94°C, 60 sec at 68°C, 3min at 72°C, 30 sec at 94°C, 60 sec at 44°C, and 3 min at 72°C. Further sequencing for the non-coding region was tried with a new gene-specific primer tim26 and int1 (Table 1). The TAIL-PCR products were digested by EcoR I, cloned into pBluescript SK(+) (Stratagene) and sequenced as described above.

Genomic DNA of *timeless* gene from *npd*-mutants was amplified by a standard PCR with a set of *timeless*-specific primers tim27, tim23 (Table 1). The PCR products were cloned into pBluescript SK(+) and sequenced as described above.

Genetic linkage analysis

Genetic linkage analysis was performed to examine the co-inheritance of the NPD phenotype (no-diapause induction in response to short days) and a mutant allele of *timeless* gene (as revealed by intron sequencing). Females of *npd*-mutant flies were mated with wild-type Sapporo strain males and the reciprocal crosses were also performed. F1 progeny were reared under long-day conditions (16h light : 8h darkness, 18°C) to prevent larval diapause and the adults were intercrossed. F2 progeny were reared under short days to screen for the NPD phenotype: those larvae that pupariated under short days (*i.e.* did not enter diapause) were recognized as *npd* individuals; those larvae that had not pupariated within 40 days after oviposition (*i.e.* entered diapause) were recognized as wild-type individuals. Later, the diapausing larvae were exposed to constant light (24h light : 0h darkness, 18°C) to terminate their diapause and allow development of adults. DNA was extracted from each individual fly and the fragment of intron-DNA in the 5'-leader sequence of *timeless* was amplified by a standard PCR with a set of primers tim24, tim29 (Table 1, Fig. 4). The PCR products were separated on 8% polyacrylamide gel by electrophoresis to analyze the length difference (a 13-bp insertion in mutated timeless allele).

RESULTS

Full-length sequence of *timeless* cDNA

The *timeless* cDNA of *C. costata* (wild-type, Sapporo strain) had 5295 nucleotides (data not shown, see EMBL/GenBank/DDBJ accession number: AB073724). It consisted of 3 main regions: 5'-leader sequence (453-bp), coding sequence (4128-bp) and 3'-untranslated region (714-bp). The sequence coded for 1355 amino acids, which corresponded to amino acids 24–1421 of *D. melanogaster*'s TIMELESS protein (Fig. 1).

Northern blot analysis

In the wild-type Sapporo strain, Northern hybridization indicated that the abundance of *timeless* mRNA transcripts oscillates on a daily basis in fly heads. The maximum abundance of mRNA transcripts was detected during the first half of the dark phase (Zt 12–15). The minimum abundance was registered during "late night" and "early morning" (Zt 0–3) (Fig. 2A). In the NPD strain, no *timeless* mRNA transcripts were detectable throughout the photoperiodic cycle (Fig. 2B).

RNAi

Double-stranded (ds) *timeless* RNA was injected into 5562 early embryos of the wild-type Sapporo strain. Another 472 embryos were injected with injection buffer (IB) alone (control-1) and 270 embryos were only treated with halocarbon oil, without any injection (control-2). Surviving larvae were reared under short-day conditions to screen their photoperiodic phenotype. Results are summarized in Fig. 3. None of the surviving control-2 larvae pupariated (all larvae entered diapause) and their *timeless* mRNA was detectable by Northern. Only about 17 or 9% of embryos survived the treatment in dsRNA injected or IB-injected (control-1) variants, respectively. Three out of 82 (3.65%) surviving control-1 larvae pupariated and remaining larvae entered diapause. The number of pupariated control-1 larvae was too low to perform a reliable Northern analysis of *timeless* mRNA (our preliminary experiments showed that 10–15 puparia is a minimum number to obtain enough total RNA). Thus, we can not decide whether the pupariation in control-1 larvae resulted from the disruption of transcription of *timeless* gene or from some non-specific effect of IB injection. Forty-six out of 504 (9.13%) surviving dsRNA-treated larvae pupariated and their *timeless* mRNA was not detectable by Northern (Fig. 3). Pupariating individuals were considered as having lost their photoperiodic response after injections with either *timeless* dsRNA or IB.

Sequence of intron in 5'-leader region of *timeless* cDNA

About 1.4Kbp genomic DNA fragment flanking to the *timeless* coding region was amplified by TAIL-PCR (Fig. 4). Comparison of intron sequences between the Sapporo and NPD strains revealed that *npd*-mutants carry a different, potentially "deficient", *timeless* allele, which we call here *timeless^{npd}*. The *timeless^{npd}* allele shows, in addition to many point-substitutions, a remarkable 13-bp insertion. This insertion sequence was used as a marker of a *timeless^{npd}* allele in a genetic linkage analysis.

Genetic linkage analysis

The *npd*-mutant flies (NPD strain), homozygous for the *timeless^{npd}* allele (with 13-bp insertion in intron), were reciprocally mated with the wild-type flies (Sapporo strain), homozygous for the normal *timeless* allele. The wild-type and *npd* photoperiodic phenotypes were distinguished based on their response to short days (diapause induction or not, respectively). Flies carrying the deficient and/or normal *timeless* alleles were distinguished by the length of PCR fragments amplified with *timeless*-intron-specific primers (218-bp or 205-bp, respectively). Pedigrees in Fig. 5a) and b) show the inheritance of photoperiodic phenotypes and the dimorphic *timeless*

Fig. 4. Alignment of intronic DNA sequences of *timeless* alleles from wild-type (Sapporo strain, Sap) and *npd*-mutant (NPD strain) of *Chymomyza costata* (alignment was performed using GENETYX-MAC software, Software Development, Tokyo, Japan). The intron is located in the 5'-leader sequence (indicated by single line above the sequence) flanking to the coding region (indicated by double line) of *timeless* gene. A remarkable 13bp-insertion found in the sequence from NPD strain (*timeless^{npd}* allele) is indicated by bold-faced letters. Primers used for PCR amplification of the insertion regions are indicated by underlines. Asterisks (*) indicate identical nucleotides.
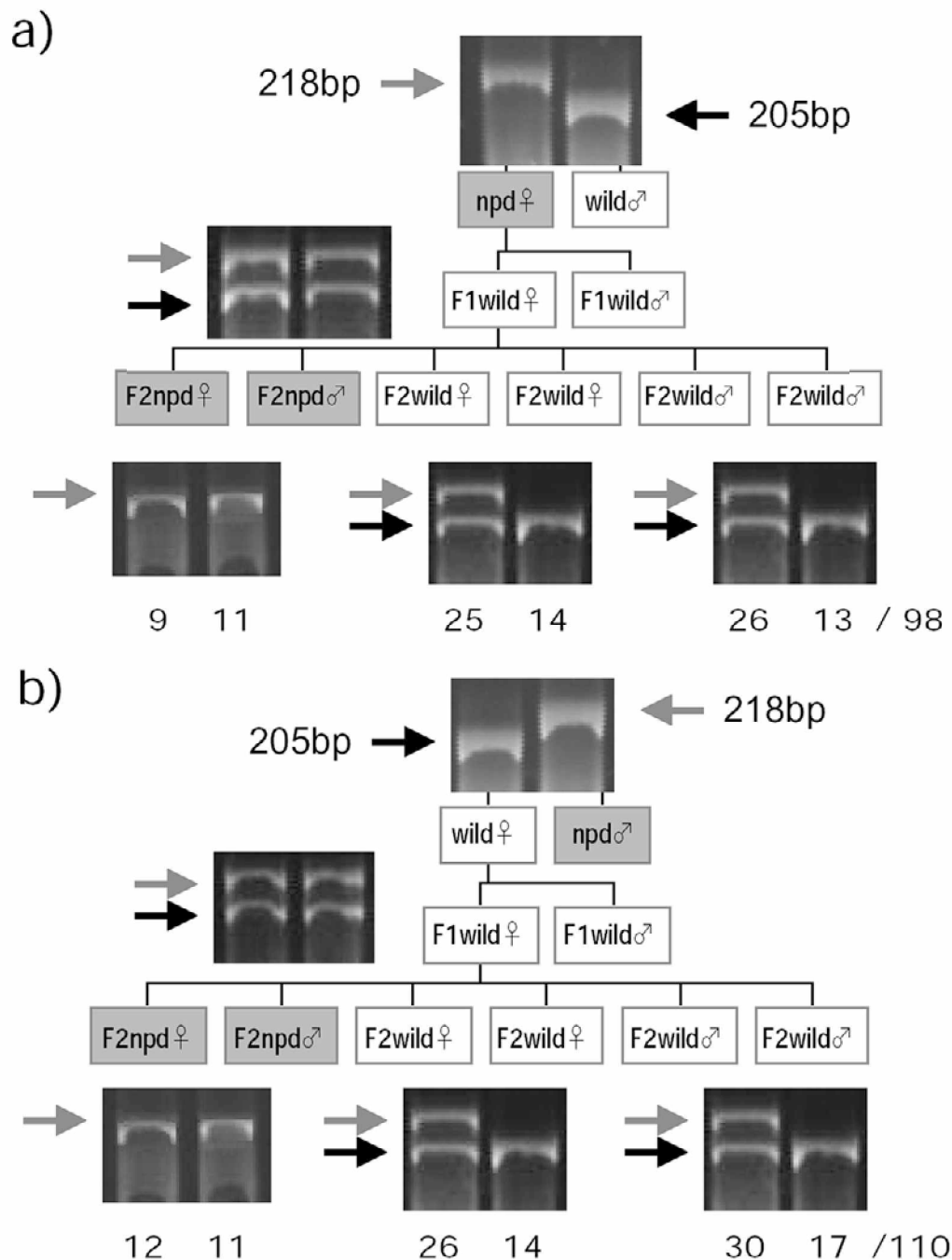
Fig. 5. Genetic linkage analysis between the photoperiodic phenotype and a dimorphic allele of *timeless* gene in the reciprocal hybrids between the wild-type flies (Sapporo strain) and *npd*-mutants (NPD strain) of *Chymomyza costata*. Photoperiodic phenotype is either wild-type (wild, white box; entrance to diapause in response to short-day signal) or *npd*-mutant (npd, gray box; no response to short-day signal). The *timeless* allele is either wild-type (yielding a 205-bp DNA fragment after PCR amplification) or *npd*-mutant type (*timeless^npd*, yielding a 218-bp DNA fragment due to the presence of a 13-bp insertion). Pedigrees show the inheritance of the two traits in the progeny of reciprocal hybrids: (a), *npd*-mutant female was mated with wild-type male; (b) reciprocal cross. Individual flies of the F2 progeny were tested for the presence of each of the two traits (numbers given below). The *npd*-mutant photoperiodic phenotype was strictly co-inherited with the *timeless^npd* allele.

alleles in F1 and F2 progeny. In both pedigrees, which reciprocally differed in parental genotypes, F1 progeny showed wild-type phenotype and carried both deficient and normal *timeless* alleles (heterozygous). In F2 progeny, which was produced by intercrosses between F1 siblings, the phenotypes and genotypes were distributed approximately according to the Mendelian laws (1:2:1 for genotypes and 3:1 for phenotypes). Importantly, a non-photoperiodic (NPD) phenotype was in all cases (21 females, 22 males) accompanied by the presence of homozygotism for a deficient *timeless^npd* allele. Individuals that were either heterozygous (51 females, 56

males) or homozygous for normal *timeless* allele (28 females, 30 males) always showed a wild-type photoperiodic phenotype.

DISCUSSION

In this study, full-length cDNA and amino acid sequences of the structural homologue of *D. melanogaster*'s *timeless* gene were obtained from a closely related fly, *Chymomyza costata*. The 62% of amino acids were identical between the two species. In several conserved domains, the structural homologuey of amino acid sequences was much higher: 88% in PER interaction domain I (PID I), 75% in PID II, and 90% in nuclear localization signal (NLS) domain. In addition, a 32 aa region that is important for restoring wild-type rhythms (Ousley et al., 1998) was also highly conserved (91% of amino acids were identical between the two species). An acidic domain, thought to be involved in the activation of transcription factor in *D. melanogaster*, is rich (60%) in acidic amino acids, E (glutamic acid) and D (aspartic acid) (Myers et al., 1995). A corresponding region in *C. costata*, however, contained only 24% acidic amino acids, and the similarity between the two species was quite low (19%). The cytoplasmic localization domain (CLD) at the C-terminus was also less conserved (49% similarity between *C. costata* and *D. melanogaster*). Reduced conservation in the CLD and acidic domains of TIMELESS was also observed among other drosophilid species (Ousley et al., 1998). The sites to which *tim^{SL}* (which shortens the wild-type period by half an hour; Rutila et al., 1996), *tim^{L1}* (lengthens the circadian rhythm; Rothenfluh et al., 2000), *tim^{L2}* (produces 26.3 h circadian rhythm; Rothenfluh et al., 2000) and *tim^{rit}* (alters free-running periods in a temperature-dependent manner; Matsumoto et al., 1999) mutations have been mapped in *D. melanogaster* were also conserved in *C. costata*.

In the wild-type (Sapporo) strain of *C. costata*, the abundance of mRNA transcripts of this gene were shown to oscillate on a daily basis, in phase with the transcripts of another clock gene, *period* (Kostal & Shimada, 2001). Similar oscillations in *period* and *timeless* transcription were found in lateral neurons of the brain of *D. melanogaster* and proved to represent a central feature of its circadian clock (Sehgal et al., 1994; Allada et al., 1998; Darlington et al., 1998; Rutilla et al., 1998; Dunlap, 1999; Harmer et al., 2001).

No mRNA transcripts, however, were detected by Northern blot analysis in the *non-photoperiodic-diapause* mutants (NPD strain) of *C. costata* (irrespective of Zeitgeber time of RNA sampling). Thus, it seems that the rate of *timeless* transcription is either zero or severely diminished in *npd*-mutants (relatively low sensitivity of Northern does not allow us to distinguish between the two possibilities). Mal-functioning of the central circadian clock system in the brain of *npd*-mutants is highly probable because: (1) no daily/circadian oscillations of *period* mRNA transcripts were found (Kostal & Shimada, 2001); and (2) no/diminished expression of *timeless* gene was detected (this study). Such a striking difference in

timeless expression between the two strains of *C. costata* appears interesting when related to *npd*-mutant's circadian and photoperiodic phenotypes. NPD strain flies are non-photoperiodic and behaviourally arrhythmic (Riihimaa & Kimura, 1988, 1989; Kostal & Shimada, 2001) and it was confirmed by classical crossbreeding experiments that both traits were caused by mutation in a single autosomal gene locus *npd* (Riihimaa & Kimura, 1989; Riihimaa, 1996; Lankinen & Riihimaa, 1992, 1997).

The above results led us to hypothesize that the *npd* locus might code for a functional element, which is shared by circadian and photoperiodic clock systems. Some results obtained in this study indicate that TIMELESS protein might be such a linking element. First, selective disruption of *timeless* gene transcription (RNAi) in the wild-type flies resulted, in a certain proportion of survivors at least, in adoption of a *npd* photoperiodic phenotype (*i.e.* no response to short days). The results of RNAi experiments, however, are difficult to interpret conclusively because of: (1) low survival rate after injection of *timeless* dsRNA (ca. 17%); (2) low rate of specific response (loss of photoperiodic sensitivity) to dsRNA (ca. 9%); and (3) relatively high non-specific response to the injection buffer alone (ca. 3.7%). All three problems (1–3) are more or less typical for RNAi methodology (Kennerdell & Carthew, 1998, 2000), especially when the phenotypic effect is to be observed some time after the injection of dsRNA. In our case, the pupariation of *C. costata* typically occurred 21 days or more after the injection, which makes the persistence of RNAi effect problematic. Second, the existence of dimorphism in the *timeless* allele (a 13-bp insertion found in intron of *npd* mutants in this study) allowed performing genetic linkage analysis of inheritance of *timeless* alleles and photoperiodic phenotype. In total, 208 F2 hybrids of Sapporo and NPD strains were individually analyzed and it was found that the deficient allele of *timeless* gene (*timeless^{npd}*) is strictly co-inherited with the non-photoperiodic phenotype.

In conclusion, introductory results presented in this paper imply that there might exist a close functional linkage between circadian and photoperiodic time measurement systems in the drosophilid fly *C. costata*. At present, the *npd* locus, which could be coding for TIMELESS protein, seems to be a candidate for such a linking element. Our ongoing work is focused on testing this hypothesis.

REFERENCES

ALLADA R., WHITE N.E., SO W.V., HALL J.C. & ROSBASH M. 1998: A mutant Drosophila homologue of mammalian clock disrupts circadian rhythms and transcription of period and timeless. *Cell* **93**: 791–804.

Bünning E. 1936: Die endogene Tagesrhythmik als Grundlage der photoperiodischen Reaktion. *Ber. Dt. Bot. Ges.* **54**: 590–607.

Darlington T.K., Wager-Smith K., Ceriani M.F., Staknis D., Gekakis N., Steeves T.D.L., Weitz C.J., Takahashi J.S. & Kay S.A. 1998: Closing the circadian loop: CLOCK-induced transcription of its own inhibitors per and tim. *Science* **280**: 1599–1603.

Denlinger D.L. 2000: Molecular regulation of insect diapause. In: Storey K.B. & Storey J.M. (eds): *Environmental Stressors and Gene Responses.* Elsevier Science, Amsterdam, pp. 259–275.

Denlinger D.L. 2002: Regulation of diapause. *Annu. Rev. Entomol.* **47**: 93–122.

Dunlap J.C. 1999: Molecular bases for circadian clocks. *Cell* **96**: 271–290.

Goldman B.D. 2001: Mammalian photoperiodic system: formal properties and neuroendocrine mechanisms of photoperiodic time measurement. *J. Biol. Rhythms* **16**: 283–301.

Goto S.G. & Denlinger D.L. 2002: Shrot-day and long-day expression patterns of genes involved in the flesh fly clock mechanism: period, timeless, cycle and cryptochrome. *J. Insect Physiol.* **48**: 803–816.

Harmer S.L., Panda S. & Kay S.A. 2001: Molecular bases of circadian rhythms. *Annu. Rev. Cell Dev. Biol.* **17**: 215–253.

Kennerdell J.R. & Carthew R.W. 1998: Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled2 act in the wingless pathway. *Cell* **95**: 1017–1026.

Kennerdell J.R. & Carthew R.W. 2000: Heritable gene silencing in Drosophila using double-stranded RNA. *Nat. Biotechnol.* **18**: 896–898.

Konopka R.J. & Benzer S. 1971: Clock mutants of Drosophila melanogaster. *Proc. Natl. Acad. Sci. USA* **68**: 2112–2116.

Kostal V., Shimada K. & Hayakawa Y. 2000a: Induction and development of winter larval diapause in a drosophilid fly Chymomyza costata. *J. Insect Physiol.* **46**: 417–428.

Kostal V., Noguchi H., Shimada K. & Hayakawa Y. 2000b: Circadian component influences the photoperiodic induction of diapause in a drosophilid fly, Chymomyza costata. *J. Insect Physiol.* **46**: 887–896.

Kostal V. & Shimada K. 2001: Malfunction of circadian clock in the non-photoperiodic-diapause mutants of the drosophilid fly, Chymomyza costata. *J. Insect Physiol.* **47**: 1269–1274.

Lakovaara S. 1969: Malt as a culture medium for Drosophila species. *Drosoph. Inf. Serv.* **44**: 128.

Lankinen P. 1986: Genetic correlation between circadian eclosion rhythm and photoperiodic diapause in Drosophila littoralis. *J. Biol. Rhythms* **2**: 101–118.

Lankinen P. & Riihimaa A.J. 1992: Weak circadian eclosion rhythmicity in Chymomyza costata (Diptera: Drosophilidae), and its independence of diapause type. *J. Insect Physiol.* **38**: 803–811.

Lankinen P. & Riihimaa A.J. 1997: Effects of temperature on weak circadian eclosion rhythmicity in Chymomyza costata (Diptera: Drosophilidae). *J. Insect Physiol.* **43**: 251–260.

Liu Y.G., Mitsukawa N., Oosumi T. & Whittier R.F. 1995: Efficient isolation and mapping of Arabidopsis thaliana T-DNA insert junctions by thermal asymmetric interlaced PCR. *Plant J.* **8**: 457–463.

Loudon A.S.I., Ihara N. & Menaker M. 1998: Effect of a circadian mutation on seasonality in Syrian hamsters (Mesocricetus auratus). *Proc. R. Soc. London* **B265**: 517–521.

Majercak J., Sidote D., Hardin P.E. & Edery I. 1999: How a circadian clock adapts to seasonal decreases in temperature and day length. *Neuron* **24**: 219–230.

Matsumoto A., Tomioka K., Chiba Y. & Tanimura T. 1999: tim^{rit} lengthens circadian period in a temperature-dependent manner through suppression of PERIOD protein cycling and nuclear localization. *Mol. Cell. Biol.* **19**: 4343–4354.

Messager S., Ross A.W., Barrett P. & Morgan P.J. 1999: Decoding photoperiodic time through Per1 and ICER gene amplitude. *Proc. Natl. Acad. Sci. USA* **96**: 9938–9943.

Myers M.P., Wager-Smith K., Wesley C.S., Young M.W. & Sehgal A. 1995: Positional cloning and sequence analysis of the Drosophila clock gene timeless. *Science* **270**: 805–808.

Nuesslein-Hildesheim B., O'Brien J.A., Ebling F.J.P., Maywood E.S. & Hastings M.H. 2000: The circadian cycle of mPER clock gene products in the suprachiasmatic nucleus of the Siberian hamster encodes both daily and seasonal time. *Eur. J. Neurosci.* **12**: 2856–2864.

Ousley A., Zafarullah K., Chen Y., Emerson M., Hickman L. & Sehgal A. 1998: Conserved regions of the timeless (tim) clock gene in Drosophila analysed through phylogenetic and functional studies. *Genetics* **148**: 815–825.

Riihimaa A.J. 1996: Genetic variation in diapause, cold-hardiness and pupal eclosion rhythm in Chymomyza costata. *Acta Univ. Ouluensis* **A274**: 1–60.

Riihimaa A.J. & Kimura M.T. 1988: A mutant strain of Chymomyza costata (Diptera: Drosophilidae) insensitive to diapause-inducing action of photoperiod. *Physiol. Entomol.* **13**: 441–445.

Riihimaa A.J. & Kimura M.T. 1989: Genetics of the photoperiodic larval diapause in Chymomyza costata (Diptera; Drosophilidae). *Hereditas* **110**: 193–200.

Rothenfluh A., Abodeeley M., Price J.L. & Young M.W. 2000: Isolation and analysis of six timeless alleles that cause short- or long-period circadian rhythms in Drosophila. *Genetics* **156**: 665–675.

Roenneberg T. & Merrow M. 2001: Seasonality and photoperiodism in Fungi. *J. Biol. Rhythms* **16**: 403–414.

Rubin G.M. & Spradling A.C. 1982: Genetic transformation of Drosophila with transposable element vectors. *Science* **218**: 348–353.

Rutila J.E., Zeng H., Le M., Curtin K.D., Hall J.C. & Rosbash M. 1996: The tim^{SL} mutant of the Drosophila rhythm gene timeless manifests allele-specific interactions with period gene mutants. *Neuron* **17**: 921–929.

Rutila J.E., Suri V., Le M., So W.V., Rosbash M. & Hall J.C. 1998: CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of Drosophila period and timeless. *Cell* **93**: 805–814.

Samach A. & Coupland G. 2000: Time measurement and the control of flowering in plants. *BioEssays* **22**: 38–47.

Sambrook J., Fritsch E.F. & Maniatis T. 1989: *Molecular Cloning, A Laboratory Manual.* 2nd ed., Cold Spring Harbor Laboratory Press, New York.

Saunders D.S. 2002: *Insect Clocks.* 3rd ed., Elsevier Science, Amsterdam, 576 pp.

Saunders D.S., Henrich V.C. & Gilbert L.I. 1989: Induction of diapause in Drosophila melanogaster: Photoperiodic regulation and the impact of arrhythmic clock mutations on time measurement. *Proc. Natl. Acad. Sci. USA* **86**: 3748–3752.

Sehgal A., Price J.L., Man B. & Young M.W. 1994: Loss of circadian behavioral rhythms and per RNA oscillations in the Drosophila mutant timeless. *Science* **263**: 1603–1606.

Shimada K. 1999: Genetic linkage analysis of photoperiodic clock genes in Chymomyza costata (Diptera: Drosophilidae). *Entomol. Sci.* **2**: 575–578.

Sumová A., Sládek M., Jác M. & Illnerová H. 2002: The circadian rhythm of Per1 gene product in the rat suprachiasmatic

nucleus and its modulation by seasonal changes in daylength. *Brain Res.* **947**: 260–270.

Suzuki L. & Johnson C.H. 2001: Algae know the time of day: circadian and photoperiodic programs. *J. Phycol.* **37**: 933–942.

Tauber E. & Kyriacou B.P. 2001: Insect photoperiodism and circadian clocks: models and mechanisms. *J. Biol. Rhythms* **16**: 381–390.

Veerman A. 2001: Photoperiodic time measurement in insects and mites: a critical evaluation of the oscillator-clock hypothesis. *J. Insect Physiol.* **47**: 1097–1109.

Wehr T.A. 2001: Photoperiodism in humans and other primates: evidence and implications. *J. Biol. Rhythms* **16**: 348–364.

Yoshida T. & Kimura M.T. 1995: The photoperiodic clock in Chymomyza costata. *J. Insect Physiol.* **41**: 217–222.