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CYCLE Is a Second bHLH-PAS Clock Protein Essential for Circadian Rhythmicity and Transcription of *Drosophila period* and *timeless*

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Summary

We report the identification, characterization, and cloning of another novel *Drosophila* clock gene, *cycle* (*cyc*). Homozygous *cyc* flies are completely arrhythmic. Heterozygous *cyc*/+ flies are rhythmic but have altered periods, indicating that the *cyc* locus has a dosage effect on period. The molecular circadian phenotype of homozygous *cyc* flies is like homozygous *Clk* flies presented in the accompanying paper: mutant flies have little or no transcription of the *per* and *tim* genes. Cloning of the gene indicates that it also encodes a bHLH-PAS transcription factor and is a *Drosophila* homolog of the human protein BMAL1. *cyc* is a nonsense mutation, consistent with its strong loss-of-function phenotype. We propose that the CYC:CLK heterodimer binds to *per* and *tim* E boxes and makes a major contribution to the circadian transcription of *Drosophila* clock genes.

Introduction

The physiological and behavioral manifestations of circadian (~24 hr) rhythms are easily observed in prokaryotic as well as eukaryotic organisms. Recent progress in a number of systems has begun to illuminate the biochemical machinery that underlies these circadian outputs. In *Drosophila* (Rosbash et al., 1996), *Neurospora* (Dunlap, 1996), photosynthetic bacteria (Kondo et al., 1994), and mice (Vitaterna et al., 1994; Tei et al., 1997) transcriptional regulation is intimately linked to the generation and maintenance of circadian rhythms: in all four systems, rhythmic transcription of clock genes appears to be part of the oscillator. Yet many clock genes do not have defined biochemical roles. In *Drosophila*, the two clock genes *period* (*per*) and *timeless* (*tim*) and their gene products are important for pacemaker activity, although their precise biochemical functions remain to be established. They are nonetheless well characterized and appear relevant to transcriptional regulation: the expression profiles of these two genes undergo robust circadian oscillations (Hardin et al., 1990, 1992; Zerr et al., 1990; Hardin, 1994; Sehgal et al., 1995; So and Rosbash, 1997) and mutations in the two proteins (PER and TIM) alter or abolish not only the periodicity and phase of behavioral rhythms but also those of their own transcription. This demonstrates that both proteins autoregulate their own transcription through a feedback loop (Hardin et al., 1990; Sehgal et al., 1995; Marrus et al., 1996). There is no evidence at present, however, that PER and TIM act directly at their own promoters to exert this transcriptional control.

There has been substantial recent progress in understanding several aspects of *per* transcription. First, work by Hardin and colleagues on the *per* promoter has identified a 69 bp element, which is capable of driving cycling transcription of a reporter gene. This enhancer contains an E box (CACGTG), a known binding site required for bHLH transcription factors (Hao et al., 1997). Second, mouse *per* genes have been identified, and at least two of these transcripts undergo circadian oscillations in mammalian tissues. Although there are features of m*per* RNA cycling that are different from those of *Drosophila per* mRNA cycling, it would not be surprising were there also critical E box-containing enhancers within the relevant mouse regulatory regions (Sun et al., 1997; Tei et al., 1997). Third, the cloning of the mouse circadian rhythm gene, *mClock*, revealed that it encodes a bHLH transcription factor; in fact, mCLOCK is a member of the bHLH-PAS family of bHLH transcription factors (Antoch et al., 1997; King et al., 1997). Thus, a reasonable model is that the bHLH region of mCLOCK may bind to these putative evolutionarily conserved E box elements and drive the cycling transcription of m*per* genes. This model is supported by two additional, very recent findings: in vitro DNA selection experiments show that mCLOCK selects DNA sequences that resemble the *Drosophila per* E box (Hogenesch et al., 1998), and we have identified a putative ortholog of m*Clock*, the product of the *Drosophila Clk* gene (CLK; Allada et al., 1998). Importantly, CLK is required for regulated *per* and *tim* transcription, consistent with the notion that this protein binds to clock gene E boxes and is required for circadian transcription in mammalian systems as well as in *Drosophila*. To the best of our knowledge, this is the only experiment that links *Clock* to a biochemical function and biochemical mutant phenotype.

In this paper we report the identification, characterization, and cloning of a novel *Drosophila* clock gene, *cycle* (*cyc*). Homozygous mutant *cyc* flies have a behavioral and molecular phenotype that resembles closely that of homozygous *Clk* flies described in the preceding paper: they are behaviorally arrhythmic and exhibit little *per* and *tim* transcription. Further phenogenetic analyses indicate that, like *Clk,* the *cyc* locus also has a dosage effect on period. They also suggest that it is a nonvital, dedicated clock gene. Remarkably, cloning of *cyc* indicates that, like *Clk*, it encodes a bHLH-PAS transcription factor and is a *Drosophila* homolog of the human gene BMAL1 (MOP3; Ikeda and Nomura, 1997; Hogenesch et al., 1997, 1998). Biochemical work from elsewhere (Hogenesch et al., 1998) indicates that BMAL1 is the partner of mammalian CLOCK and that the heterodimer binds to and activates transcription from *per*-like E boxes. Based on all of these results, we propose that the CYC:CLK heterodimer binds to *per* and *tim* E boxes and makes a major contribution to the circadian transcription of *Drosophila* clock genes.

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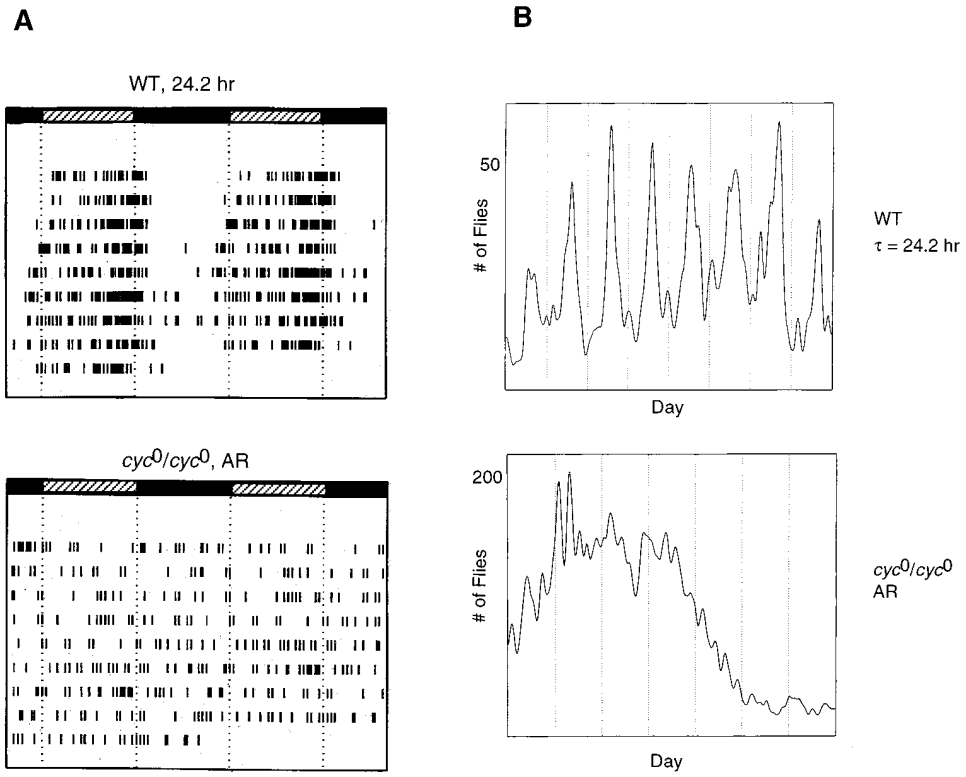

Figure 1. cyc^0/cyc^0 Flies Are Arrhythmic

(A) Examples of locomotor activity plots of two individual flies, one Canton-S wild-type (WT) and one cyc^0/cyc^0, in constant darkness (dark:dark, DD). Each hash mark is the equivalent of ten activity events. The data are double plotted, where activity from day 1 and 2 is on the first line, day 2 and 3 on the second, and so on. The dark bars indicate the subjective night, and the hashed bars, the subjective day. The period (τ), determined by χ^2 analysis, is shown above each graph (AR, arrhythmic). These two plots are representative of what flies from their respective genotypes look like.

(B) Eclosion profiles of WT and cyc^0/cyc^0 flies in DD. The number of flies eclosing from their pupal cases is plotted as a function of time (N = 1500 for WT, N = 5300 for cyc^0). Vertical lines indicate the end of the subjective night, and data for 7 or 8 days is shown. The period calculated by χ^2 analysis for each genotype is given (AR, arrhythmic).

Results

cyc Is a Novel Arrhythmic Mutant, Abolishes PER and TIM Cycling, and Markedly Reduces Protein Levels

As described in the accompanying paper (Allada et al., 1998 [this issue of *Cell*]), we searched for novel *Drosophila* circadian rhythm genes by chemically mutagenizing flies and screening for altered or aberrant locomotor activity rhythms. A recessive arrhythmic mutant fly line was identified, out of 3170 lines screened (Rutila et al., 1996), which we have named *cycle* (cyc^0). These flies show arrhythmic locomotor activity patterns when they carry two mutant third chromosomes (Figure 1A). cyc^0/cyc^0 flies also manifest arrhythmic eclosion (Figure 1B). The homozygous mutant flies are always arrhythmic, regardless of the *per* genetic background (Table 1). Unlike the semidominant *Clk* mutant, heterozygous $cyc^0/+$ flies manifest robust rhythms with no hint of arrhythmicity. But they have altered periods, with rhythms 1 hr longer than their *per* genotype would otherwise dictate (Table 1). This indicates that either *cyc* has a dominant phenotype or that the locus has dosage sensitive effects on period. The data suggest that *cyc* might identify a circadian clock component.

Homozygous mutant cyc^0/cyc^0 flies also have difficulties under 12:12 light/dark (LD) conditions, since only 64% of them give discernable rhythms by χ^2 analysis (Table 2). It has been previously shown that >90% of arrhythmic per^0 and tim^0 flies are light responsive and therefore show robust 24 hr rhythms under these conditions (Wheeler et al., 1993; Table 2). As the cyc^0/cyc^0

Table 1. cyc^0 Causes Arrhythmicity or Altered Circadian Periods

Genotype	Period ± SEM[a]	% AR[b]	N[c]
WT[d]	24.2 ± 0.1	0	30
per^L	28.8 ± 0.2	19	21
per^S	19.3 ± 0.1	0	7
per^+; cyc^0/cyc^0	AR	100	141
per^+; $cyc^0/+$	25.0 ± 0.1	0	30
per^L; cyc^0/cyc^0	AR	100	27
per^L; $cyc^0/+$	30.2 ± 0.3	17	30
per^S; cyc^0/cyc^0	AR	100	10
per^S; $cyc^0/+$	19.9 ± 0.1	9	11

[a] Period of activity in constant darkness, given in hours ± SEM.
[b] Percentage of flies that are arrhythmic (AR) by X^2 analysis, $\alpha = .01$.
[c] Number of animals tested.
[d] Wild-type = Canton-S.

Table 2. *cyc*^{0} Flies Do Not Properly Entrain to Light:Dark Cycles

Genotype	Period ± SEM[a]	% AR[b]	N[c]
WT	24.0 ± 0.0	0	32
tim^0	24.2 ± 0.1	9	32
cyc^0	26.6 ± 3.5	36	103

[a] Period of activity in 12 hr light:12 hr dark cycling conditions given in hours ± SEM.
[b] Percentage of flies that are arrhythmic (AR) by X^2 analysis, $\alpha = .01$.
[c] Number of animals tested.

flies show no visual difficulties in optomotor behavior (Table 3), the data suggest that the flies are specifically impaired in circadian light perception. A similar phenotype is seen with *Clk* mutant flies (Allada et al., 1998).

To examine clock function more directly, we assayed the fluctuations of the clock proteins PERIOD (PER) and TIMELESS (TIM) in wild-type, heterozygous, and homozygous *cyc* flies under LD conditions (Figure 2). Western analysis with an anti-PER antibody reveals very little protein in *cyc*^{0}/*cyc*^{0} fly heads at any time of day. As predicted from the robust rhythms, *cyc*^{0}/+ heterozygotes show normal PER cycling, with normal levels and a normal temporal phosphorylation program (Edery et al., 1994; compare *cyc*^{0}/+ to wild-type controls; Figure 2). For all genotypes, similar results were obtained for TIM (data not shown).

Low PER and TIM Levels Are Due to Low Transcription Rates

The low PER and TIM levels could be due to reduced protein stability or to reduced protein synthesis in the homozygous mutant strain. To distinguish between these possibilities, we measured *per* and *tim* RNA levels. The RNase protection assays (Figures 3A and 3B) revealed low RNA levels and little or no cycling in the *cyc*^{0}/*cyc*^{0} head extracts, suggesting reduced synthesis rather than reduced stability. As expected, RNA levels and cycling in *cyc*^{0}/+ heterozygotes were indistinguishable from those in wild-type extracts (Figures 3A and 3B).

The *cyc* effect on *per* and *tim* RNA levels and cycling could be transcriptional or posttranscriptional. To directly measure transcription rates, we performed nuclear run-on assays in homozygous *cyc* flies. In this genotype, *per* and *tim* transcription rates showed no evidence of cycling and were approximately equal to the very low trough levels of wild-type flies observed at ZT1 (Figure 3C). The result is essentially identical to that observed in homozygous *Clk* flies (Allada et al., 1998).

Table 3. *cyc*^{0} Flies Are Not Blind by Optomotor Testing

Genotype	Optomotor Score[a]	N[b]
WT	66.9 ± 2.7	14
omb^{H31}	43.5 ± 2.5[c]	15
cyc^0	68.8 ± 4.1	10

[a] This behavioral response score indicates how well the flies move with respect to moving visual stimuli (Stanewsky et al., 1996).
[b] Number of animals tested.
[c] A negative control mutant (optomotor blind) that moves arbitrarily with respect to the moving visual stimuli (Stanewsky et al., 1996).

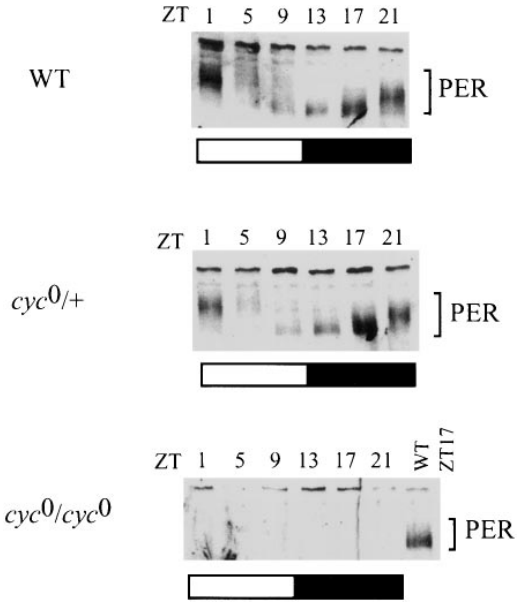

Figure 2. PER Is Dramatically Reduced in *cyc*^{0}/*cyc*^{0} Flies

Flies were collected at the indicated times during a light:dark (LD) cycle. Western blots of head extracts were reacted with anti-PER antibody. The open bars indicate the lights-on phase, and the dark bars indicate lights-off. These results have been repeated five times, and in some blots a low amount of noncycling PER was observed (J. R. and N. E. White, unpublished data).

It is interesting to note that the transcription rates in both genotypes are much lower than those observed in either *per*^{0} or *tim*^{0} head extracts (Figure 3D), which also manifest little or no transcriptional oscillations (Hardin et al., 1990; Sehgal et al., 1994; Hunter-Ensor et al., 1996). This suggests that *cyc* as well as *Clk* are epistatic to and upstream of *per*^{0} and *tim*^{0}. Taken together, the data suggest that *cyc*, like *Clk*, affects the transcription of the clock genes *per* and *tim*.

To identify specific sequence elements mediating the mutant effects on transcription, we assayed the effects of the *cyc* mutation on a minimal *per* promoter element. This 69 bp enhancer contains a critical *per*-derived E box and drives rhythmic expression of a reporter gene (*lacZ*; Hao et al., 1997). To this end, we crossed the E box/*lacZ* construct into *cyc*^{0}/*cyc*^{0} mutant flies and assayed *lacZ* RNA levels for cycling by RNase protection (Figure 3E). The results are dramatic and indicate that there is little or no cycling *lacZ* RNA transcription in the homozygous mutant flies (Figure 3F), suggesting that CYC affects the transcriptional activity of the *per* circadian transcriptional enhancer. These features of CYC are similar to those of CLK, which probably binds to and activates transcription at the *per* E box (Allada et al., 1998).

cyc Maps to 76C on Chromosome 3

Our preliminary meiotic mapping of the *cyc* mutation placed it on the left arm of the third chromosome (3L). As the arrhythmic *Clk* mutant has similar phenotypic characteristics and is also on 3L (Allada et al., 1998), we verified that they are genetically separable: 3 *cyc*$^+$,

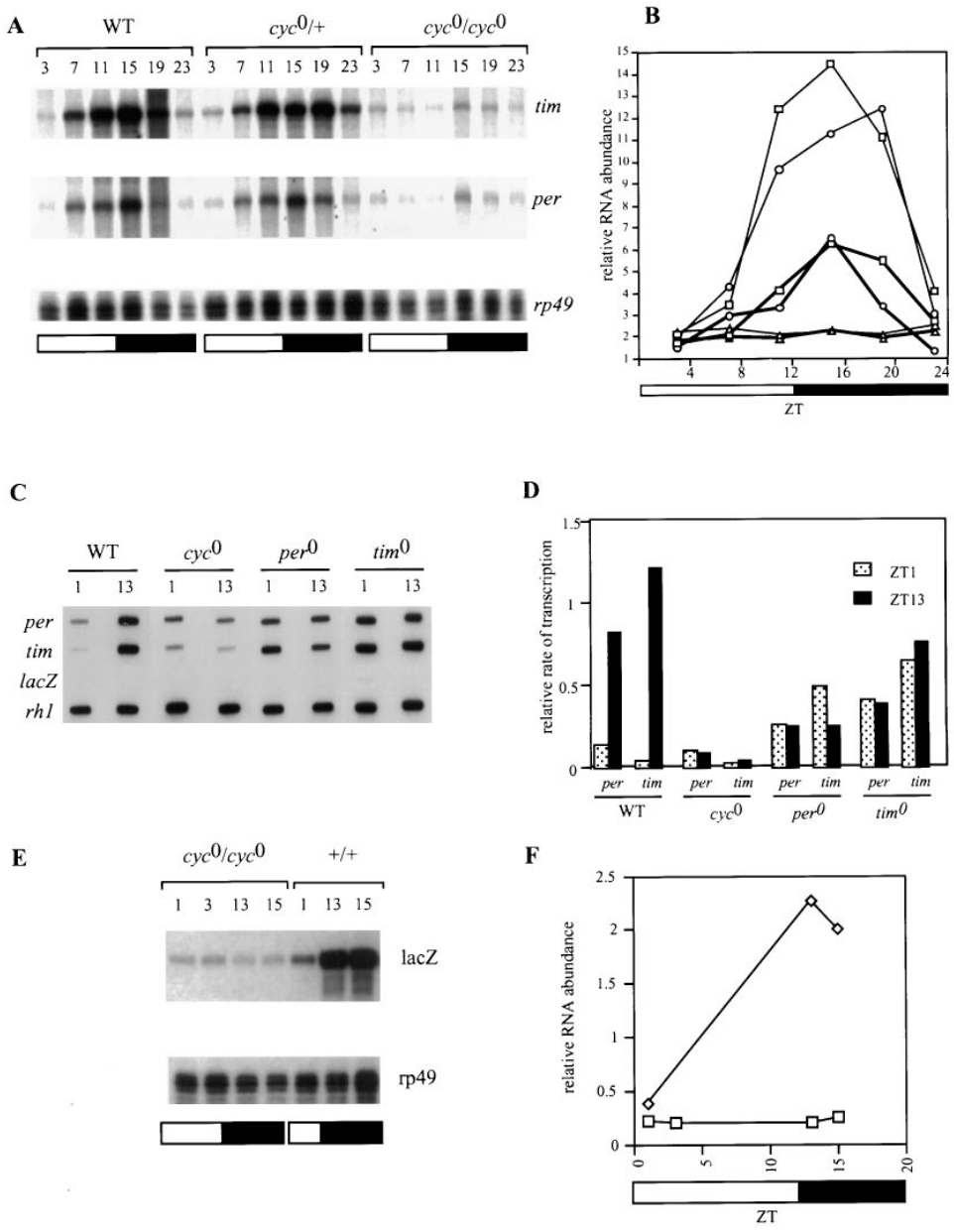

Figure 3. *per* and *tim* RNA Is Reduced and Does Not Cycle in cyc^0/cyc^0 Flies

(A) An RNase protection of head extracts, collected at various times of day during an LD cycle. The protected fragments for *tim*, *per* and rp49 (a noncycling control) are shown. The light cycle is indicated by the open (light) and filled (dark) bars. The RNA levels in cyc^0 flies was repeated twice.

(B) Quantitation of the RNase protection shown in (A). Thick lines refer to normalized *per* RNA amounts, and thin lines refer to normalized *tim* RNA amounts. The squares indicate Canton-S wild type (WT), circles indicate $cyc^0/+$, and triangles indicate cyc^0/cyc^0.

(C) A blot of newly synthesized RNAs from a nuclear run-on assay. Head extracts were collected from WT, cyc^0, per^0, or tim^0 at ZT1 (during the light) or ZT13 (during the dark). Nascent, labeled RNAs were then used as probes on blots containing *per*, *tim*, *lacZ* (a negative control), or *rh*1 (a noncycling control) DNA.

(D) Quantitation of the nuclear run-on blot. *per* and *tim* RNA amounts were normalized to *rh*1. Light bars show relative rates of transcription at ZT1, and dark bars, at ZT13.

(E) cyc^0/cyc^0 abolishes cycling from a minimal *per* promoter element. All flies contained one copy of a *per* −563 to −494/*hs*/*lacZ* transgene (Hao et al., 1997). The genotype at the *cyc* locus is indicated. Heads were isolated at the indicated times and used in an RNase protection assay. The reporter *lacZ* and the rp49 control bands are shown.

(F) Quantitation of the RNase protection shown in (E). The amount of *lacZ* RNA is normalized to the amount of rp49 RNA. Diamonds show relative RNA abundance in wild-type flies, and squares, in cyc^0/cyc^0 mutant flies.

Table 4. A Deficiency in 76B-D Fails to Complement cyc^0 and Has a Dosage Effect on Circadian Period

Genotype	Period ± SEM[a]	% AR[b]	N[c]
WT	24.2 ± 0.1	0	30
cyc^0/cyc^0	AR	100	141
cyc^0/*Df(3L)kto2*	AR	100	7
$cyc^0/+$	25.0 ± 0.1	0	30
Df(3L)kto2/+	25.0 ± 0.1	27	11

[a] Period of activity in constant darkness, given in hours ± SEM.
[b] Percentage of flies that are arrhythmic (AR) by X^2 analysis, $\alpha = .01$.
[c] Number of animals tested.

Clk^+ recombinants were obtained from 20 descendants of *Clk/cyc* females (N. E. White and J. C. H., unpublished data). Further meiotic mapping placed *cyc* to the right of *scarlet* and to the left of *radius incompletus* (*ri*). Using deletions located within this region of chromosome 3, we found that the deletion *Df(3L)kto2* uncovered cyc^0 as assayed behaviorally (Table 4), indicating that the mutation lies within the deletion interval. It also uncovered the molecular phenotype, as *Df(3L)kto2*/cyc^0 flies had low and noncycling *per* mRNA levels (data not shown). Importantly, this homozygous lethal deficiency shows a clear period-lengthening effect when assayed as a heterozygote (*Df(3L)kto2*/+; Table 4). The period (25 hr) is identical to that of $cyc^0/+$ flies. Moreover, the behavioral phenotype of cyc^0/cyc^0 is indistinguishable from that of *cyc0/Df(3L)kto2* (Table 4), suggesting that the cyc^0 mutation is a null mutation. This also suggests that the $cyc^0/+$ phenotype is not due to a dominant effect of the mutation but to a dosage effect of the locus on period.

cyc Encodes a Novel bHLH-PAS Protein

To clone the *cyc* gene product, we started to analyze five YAC clones that span the relevant genetic interval. We also began a search for candidate gene products, based on the similar phenotypic effects of *Clk* to those of *cyc* (Allada et al., 1998). Since most bHLH-PAS proteins function as heterodimers with other bHLH-PAS proteins (Crews, 1998), we hypothesized that *cyc* might encode a CLK partner and, therefore, a novel *Drosophila* bHLH-PAS protein. BLAST searches were then used to identify potential bHLH-PAS proteins in the *Drosophila* EST database (Berkeley *Drosophila* Genome Project/HHMI EST Project, unpublished data). Primers were made from each of the EST sequences and used for PCR with the five YAC clones as templates. Two of the YACs, DYN09–46 and DYN09–31, which overlap from 76C1 to 76D5, gave a product with primers from the EST GM02625 (Figure 4). Sequencing of the entire EST and comparisons with sequence databases confirmed that the gene encodes a bona fide bHLH-PAS protein. It is very closely related (P value = e^{-108}) to the mammalian gene *bmal1*, a bHLH-PAS protein enriched in brain and muscle (Ikeda and Nomura, 1997; Figure 5A). The EST was derived from an ovarian library (Berkeley *Drosophila* Genome Project/HHMI EST Project, unpublished data). To be certain that the gene is expressed in tissues more relevant for circadian rhythms, a *Drosophila* head library was screened. A cDNA of similar size was isolated, and this cDNA contained all of the EST coding sequence (J. R., unpublished data).

In order to identify the cyc^0 mutation, we directly sequenced RT–PCR products from either cyc^0 or control (nonmutant line from same mutagenesis experiment) polyA$^+$ RNA using unique 3′ primers. A single base pair difference was found: the nucleotide at position 586 is an A in wild type and a T in cyc^0. This results in a change from the wild-type lysine at amino acid 159 to a stop codon in cyc^0 (Figure 5B), which should eliminate the C-terminal 60% of the protein, including all of the PAS B domain. Such a major change to the integrity of the

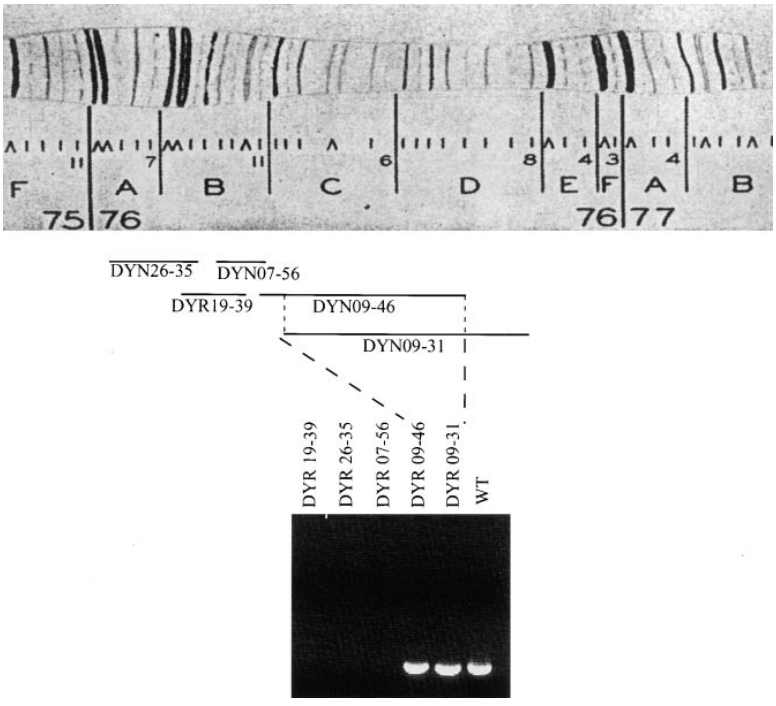

Figure 4. A bHLH-PAS Protein Maps to 76C/D in Chromosome 3

YAC clones that span the chromosomal region deleted in *Df(3L)kto2* are diagramed relative to the cytogenetic map. When primers from the EST clone GM02625 were used for PCR, two of these YACs, DYR09–46 and DYR09–31, were able to give a product of the same size as wild-type genomic DNA (~500 bp). The ethidium-stained 1% agarose gel separating the PCR products is shown.

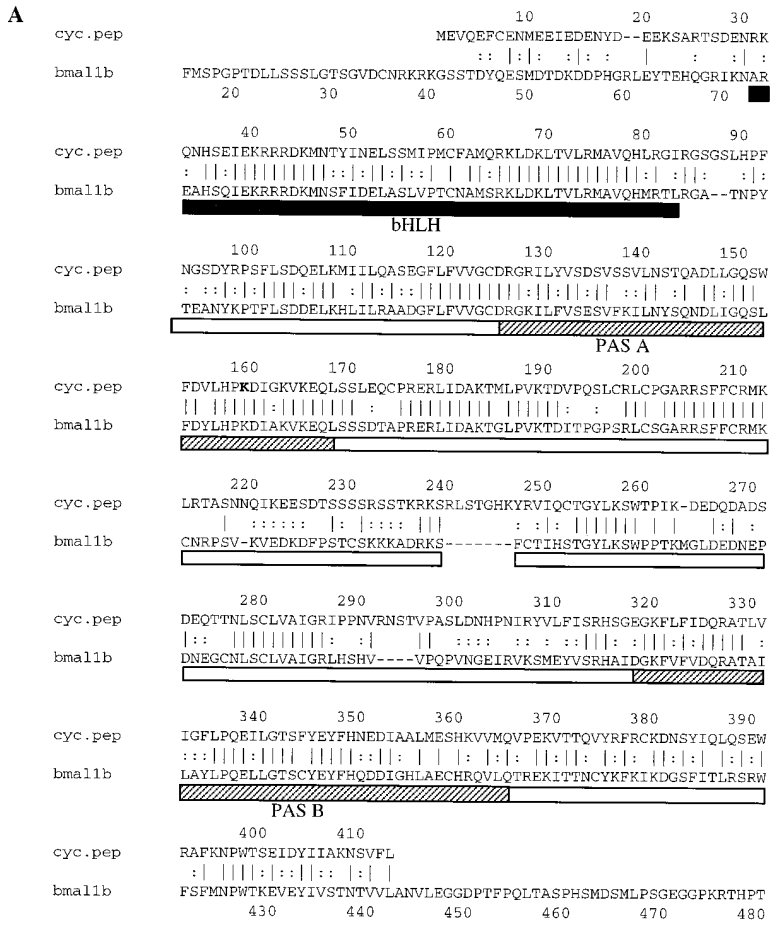

Figure 5. *cyc* Encodes a Novel bHLH-PAS Protein and Is Highly Homologous to *bMal1*

(A) The entire coding sequence of *cyc* is shown aligned to *bMal*1b. Identical amino acids are indicated by lines, and similar amino acids, by dots. Overall, *cyc* shows 52.2% identity over 412 amino acids using the FASTA algorithm (Pearson and Lipman, 1998) in the GCG package (Genetics Computer Group, WI). The basic helix-loop-helix region is indicated by the filled box. The PAS regions are shown by the open box, and the PAS A and PAS B repeats are shown by the hashed boxes. The lysine in *cyc* at amino acid 159, which is changed to a stop codon in cyc^0, is indicated in bold. The *bMal*1b coding sequence continues to amino acid 626 (Ikeda and Nomura, 1997).

(B) The cyc^0 mutation is a nonsense mutation at amino acid 159. Diagram of *cyc* and *bMal*1, showing the regions of homology. The dark bars show the bHLH, PAS A, and PAS B repeats. The PAS region is shown in gray. The K159 to stop mutation in cyc^0 is also shown.

bHLH-PAS region is consistent with the apparent loss-of-function phenotype. Although definitive verification that this *bmal1* homolog is indeed *cyc* awaits transgene rescue, the genetic mapping and mutation identification provide strong evidence that the premature stop codon at this location in cyc^0 flies is responsible for the mutant behavior.

To examine *cyc* mRNA, we assayed Northern blots of polyA$^+$ RNA using the EST sequence as a probe. Only a single mRNA was apparent, and it exhibited little or no temporal cycling (Figure 6). Also, the transcript from cyc^0/cyc^0 RNA migrates reproducibly more slowly than RNA from wild-type flies (Figure 6; data not shown). In both yeast and mammals, premature stop codons have been shown to circumvent polyA shortening during RNA turnover, leading to mRNA with longer polyA tails (Shyu et al., 1991; Muhlrad and Parker, 1994). The slower migration of the cyc^0 RNA is therefore consistent with the presence of a stop codon. We conclude that *cyc* encodes the *Drosophila* homolog of *bmal1* and that this bHLH protein is important for the transcription of circadian rhythm genes.

Discussion

The cloning of the *Drosophila* gene defined by the *cyc* circadian rhythm mutation has identified a putative transcription factor of the bHLH-PAS family. Remarkably, this novel rhythm gene is the third PAS-containing clock protein in this organism (Huang et al., 1993; Allada et al., 1998), making this motif a cornerstone of circadian rhythms not only in *Drosophila* but also in mammals and perhaps even *Neurospora* (Antoch et al., 1997; King et al., 1997; Crosthwaite et al., 1997).

The fly protein has striking similarity to a mammalian bHLH-PAS protein called BMAL1 or MOP3 (Ikeda and

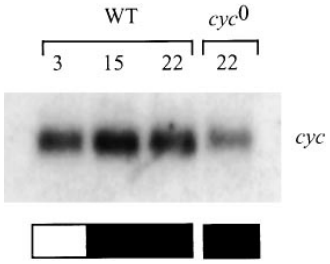

Figure 6. The *cyc* Transcript Does Not Cycle

A Northern blot of head extracts, taken at the indicated times and probed with *cyc,* is shown. The transcript migrates at ~1.7 kb and shows no discernable cycling in this and two other experiments. The slightly higher migrating *cyc* transcript seen in cyc^0 flies was also reproduced, although the lower abundance of the cyc^0 transcript was not.

Nomura, 1997; Hogenesch et al., 1998). The P value from this BLAST search is much better than that from the next closest relative, the bHLH-PAS protein ARNT (P value = e^{-108} versus e^{-75}; see below). The conservation is extensive throughout almost the entire 414 amino acids (55% identity; 68% similarity); BMAL1b then has an additional ~200 C-terminal residues. The conservation only breaks down in two regions: the ~30 N-terminal amino acids, after which the conserved bHLH region begins, and two rather large gaps between the PAS A and PAS B repeats (Figure 5A). It should be noted that the *bmal1* gene appears to code for a variety of protein products, some of which resemble CYC (Ikeda and Nomura, 1997).

BMAL1 was cloned as an "orphan" protein of the bHLH-PAS transcription factor family with no known biological function (Ikeda and Nomura, 1997; Hogenesch et al., 1997). However, there are recent biochemical experiments indicating that it may play a role in circadian rhythm–relevant transcription in mammals (Hogenesch et al., 1998): it can act as a heterodimeric partner of mCLOCK in DNA binding and transcriptional activation, the BMAL1:mCLOCK heterodimer selects a DNA-binding sequence that resembles the critical E box sequence within the cycling element in the *Drosophila per* upstream region, and there are features of this enhancer in addition to the central CACGTG hexamer that provide specificity for the BMAL1:mCLOCK heterodimer. As transcripts from mouse *per* genes undergo circadian oscillations in level (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997; Sun et al., 1997; Tei et al., 1997), these genes may contain a similar target cycling element to that of *Drosophila per*. The BMAL1:mCLOCK heterodimer could be the heterodimeric factor that binds to this cycling element and activates clock-relevant transcription (Hogenesch et al., 1998).

By analogy, we propose that CYC and CLK heterodimerize, bind to *Drosophila* clock gene E boxes, and function to drive circadian-regulated transcription of these genes (Figure 7). This makes *cyc* and *Clk* the first *Drosophila* circadian rhythm genes with a known biochemical role and a defined place in the clock circuit; it also places them upstream of *per* and *tim*. Our RNA and transcription experiments in cyc^0 and in *Clk* mutant

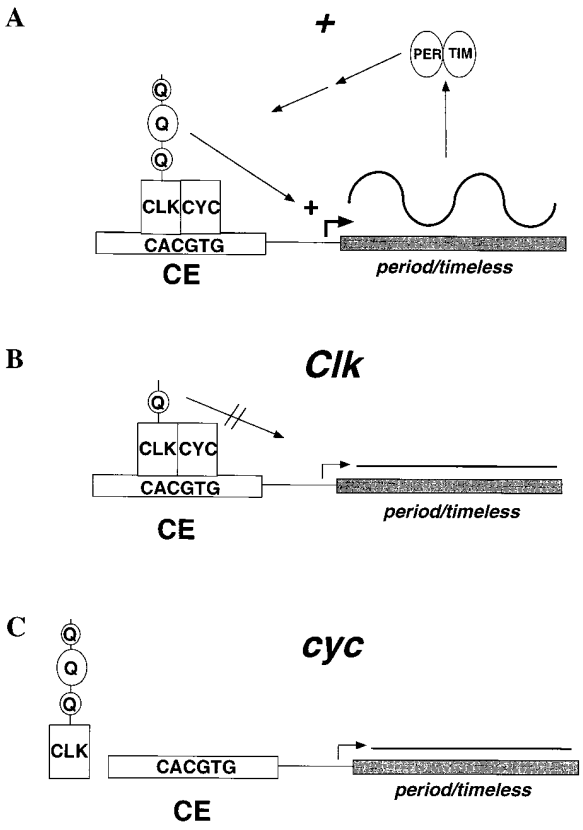

Figure 7. Model of CLOCK and CYCLE Function

(A) CLK and CYC in a wild-type fly. CLK has polyglutamine repeats (Q) and, with CYC, is able to bind the CACGTG E box within the circadian enhancer (CE) for both the *per* and *tim* promoters. The strong arrow refers to the initiation of strong cycling transcription of the *per* and *tim* genes.

(B) When the fly is mutant at the *Clk* locus, CLK and CYC can still bind but cannot activate cycling transcription of *per* and *tim* (Allada et al., 1998).

(C) If the fly is cyc^0, we assume that CLK is unable to bind to the CE, and low-level, noncycling *per* and *tim* transcription results.

flies (Figure 3; Figure 4 of Allada et al., 1998) fully support such an assignment.

CYC is approximately half as big as CLK, and the difference appears to be largely the extensive glutamine-rich C-terminal half of CLK (Allada et al., 1998). This may indicate that CLK brings the transcriptional activation domain to the complex. The dominant phenotype of the *Clk* mutant and the elimination of the Q-rich region by the mutation (Allada et al., 1998) are consistent with this notion. The mutant protein would then be able to dimerize with CYC and bind DNA but would be unable to activate transcription. This would explain its recessive as well as its dominant features.

In the case of *cyc*, the heterozygous $cyc^0/+$ phenotype is a one hour period lengthening with no obvious molecular correlates (Table 1; Figures 2 and 3A), although we would miss subtle changes in transcript or protein profiles with 4 hr time points. The *cyc* deletion/+ phenotype is identical (Table 3), indicating that the $cyc^0/+$ phenotype is not due to a dominant mutant effect but rather to a dosage-sensitive effect on period. A similar

phenotype is manifest by *per*$^{-}$ and *Clk*$^{-}$ deletions (Smith and Konopka, 1982; Allada et al., 1998). The phenotype of *cyc* and *Clk* heterozygous deficiency flies presumably reflects a reduced level of functional heterodimer. As a consequence, it takes longer for target gene products (like PER and TIM) that are important for pacemaker function to reach a required level. It then takes longer to complete a cycle, resulting in a longer rhythm.

The early stop codon in *cyc*, as well as the fact that the heterozygous *cyc*^{0}/+ phenotype is identical to that of deletion/+, indicates that *cyc* is a complete loss-of-function mutation and that the locus is nonvital. This may be a general feature of clock genes. The absence of the gene product also underlies the homozygous mutant phenotype: behavioral arrhythmicity and the almost complete absence of detectable *per* and *tim* transcription. This suggests that *cyc*, like *per* and *tim* (Konopka and Benzer, 1971; Sehgal et al., 1994), is an inessential gene that is apparently dedicated to circadian clock function. Given the nature of the gene product and the defective *per* and *tim* transcription, the lack of clock gene transcription is probably the cause of the arrhythmicity. The *cyc* genetic data are unique as there is no comparable functional information on BMAL1 in mammalian systems.

Although the *cyc* phenotype suggests that it encodes a dedicated circadian rhythm factor, the mammalian data suggest that this may not necessarily be the case. BMAL1 appears to function in concert with other proteins in addition to CLOCK (Hogenesch et al., 1998). One of these is NPAS2 (also called MOP4), which is very closely related to mCLOCK. Whereas NPAS2 might also have a circadian rhythm–restricted phenotype, other BMAL1-interacting proteins include HIF1α (Hogenesch et al., 1998), a protein that mediates cellular responses to low oxygen and glucose. Therefore, BMAL1 in mammals and CYC in flies might have several partner proteins, only some of which have dedicated circadian rhythm functions. Consistent with this notion, the next closest CYC relative after BMAL1 is ARNT (aryl hydrocarbon nuclear translocator). It is a founding member of the PAS family and was originally identified as the partner of AH, the aryl hydrocarbon receptor (Burbach et al., 1992). ARNT in mammals and its apparent ortholog TANGO in flies have several other partners (Sonnenfeld et al., 1998). Therefore, CYC as well as BMAL1 may also have multiple partners in vivo, consistent with a possibly wider role than just circadian rhythms. A confounding consideration is the presence of multiple genes in mammals, e.g., m*Clock* and *npas2*, with similar but not necessarily identical functions. This is less frequent in flies, where there is often only a single or a more restricted number of family members (Miklos and Rubin, 1996). Therefore, BMAL1 might have multiple partners in mammals and CYC only one in flies. *Clk* is also likely to be an inessential, dedicated rhythm gene (Allada et al., 1998). This might even be the case in mammals, as the mouse mutant phenotype is similar to that of *Clk* in flies (Vitaterna et al., 1994). But the genetic data also dictate caution: the mutations in both systems are dominant, indicating that the knockout or homozygous null mutant phenotype is unknown.

There is no evidence that *cyc* transcripts undergo circadian oscillations in level (Figure 6; data not shown). But low amplitude cycling could have easily escaped our attention, especially if there is cycling restricted to specific tissues in the brain. A similar negative result exists for *Clock* mRNA cycling in mouse systems (Tei et al., 1997). This suggests that there is probably significant circadian regulation of CYC activity, and it will be critical to analyze *cyc* expression at the protein level. It is attractive to postulate that PER, another *Drosophila* PAS rhythm protein, interacts with CYC and/or CLK to regulate the activity of the heterodimer in a circadian manner (Figure 7). Other less-direct modes of regulation are possible.

It is remarkable that these novel *Drosophila* rhythm genes, *Clk* and *cyc*, should not only be identified in similar genetic screens but characterized and cloned at the same time. We were aware of the importance of regulated transcription of *per* and *tim* to the rhythmic phenotype and assayed all putative arrhythmic mutants (25 in total; J. R., unpublished data) for the presence of rhythmic *per* and *tim* expression. Two mutants, *cyc* and *Clk*, were strikingly similar and had low and temporally constant levels of protein, mRNA, and *per* and *tim* transcription. The parallels with mammalian *Clock* as well as with its apparent partner *bmal1* indicates that a highly conserved and ancient transcriptional circuit is a central feature of circadian rhythms in a wide range of contemporary organisms.

Experimental Procedures

Flies

per^{L};*ry*^{506} flies were used for mutagenesis, and *per*^{L};*ry*^{506}, *per*^{s};*ry*^{506}, *per*^{0};*ry*^{506}, *tim*^{0};*ry*^{506}, and wild-type Canton-S flies were used as behavioral and molecular controls. The optomotor assay used *omb*^{H31} flies as the optomotor-blind control (Stanewsky et al., 1996). The deletion stock *Df(3L)kto2* and all mapping stocks, including the P element line *l(3)j4E61* (Spradling et al., 1995), were obtained from the Bloomington Stock Center. The transgene that contains the minimal *per* promoter element from −563 to −494, a basal heat-shock promoter, and a *lacZ* reporter gene was kindly provided by P. Hardin (Hao et al., 1997).

Fly Mutagenesis

The mutagenesis was carried out as described previously (Rutila et al., 1996). Briefly, *per*^{L};*ry*^{506} males were fed EMS, 3710 lines were constructed that contained one or two copies of mutagenized third chromosomes, and four flies from each line were assayed for alterations in locomotor activity rhythms. The *cyc*^{0} line was identified by four individuals being arrhythmic. Rough mapping of the *cyc*^{0} mutation verified that it resides on the third chromosome. All *cyc*^{0} lines assayed here contained the eye marker *ry*^{506}.

Behavioral Analyses

Flies were entrained for two 12 hr light:12 hr dark (12:12 LD) cycles before being assayed for locomotor activity in constant darkness at 25°C for 5 days (Hamblen et al., 1986). Activity periods were determined by χ^2 periodogram analysis, $\alpha = 0.01$. Alternatively, flies were kept in 12:12 LD cycles for 5 days at 25°C and periods were calculated from these LD data. Eclosion rhythms were monitored at 18°C for 7–8 days in a TriKinetics monitoring system as described previously (Konopka et al., 1994), and periods calculated from pooling three experiments by χ^2 analysis, $\alpha = 0.05$. Optomotor testing was performed as in Stanewsky et al. (1996). Activity indices (vigor of behavior in these tests) are set to 1.00 for wild type and were 1.22 for *cyc* and 0.99 for *omb*.

Mapping of the *cyc* Mutation

cyc was first found to map to the right of *scarlet* (*st*, chromosome 3, map position 44, cytological location 73A3–4): 1 out of 62 recombinants was *st*, *cyc*, and *ry* (3-52, 87D6–13). This map position was verified by mapping *cyc* with respect to the P element–defined lethal *l(3)j4E6*[1] (75E3–5): 4/481 flies were recombinants containing *l(3)j4E6*[1], *cyc*, and *ry*. The marker *ri* (3-46, 77E-F) was mapped to the right of *cyc*: 5/67 flies were recombinant between *cyc* and *ri*, and 4 of those recombinants had *ry* segregating with *ri*. Further mapping with deletions identified one, *Df(3L)kto2* (76B1–2;76D5), which did not complement *cyc* (see Results).

Molecular Analyses

For all molecular analyses, flies were collected on dry ice at the indicated time points after being entrained to a 12:12 LD cycle for 3 days. Western blotting was performed as in Edery et al. (1994). One third of a 30 head sample was run on a 6% SDS-polyacrylamide gel (29.6:0.4 acrylamide:bisacrylamide). The antibody used was an anti-PER antibody made in rabbits (Zeng et al., 1996). RNase protection assays were performed as in Marrus et al. (1996) and Hao et al. (1997) using either 30 heads (Figure 3A) or 50 heads (Figure 3E) per lane. Nuclear run-on analysis was performed as in So and Rosbash (1997). All gels were quantitated by phosphoimager using Molecular Analyst software (Bio-Rad). Northern analysis used 1 μg of polyA$^+$ RNA loaded per lane on a 1% agarose formaldehyde gel (Rouyer et al., 1997). The probe for the Northern shown in Figure 6 was an ~600 bp genomic PCR fragment that spans the upstream untranslated region and part of the bHLH region of GM02625 (see below). The blot was exposed to X-ray film with an intensifying screen for 22 hr.

Identification of the *cyc* Mutation

PCR primers were made corresponding to nucleotides 40–68 and 304–285 (GenBank, accession number AF065473) from the Expressed Sequence Tag (EST) clone GM02625 (Berkeley *Drosophila* Genome Project/HHMI EST Project, unpublished data). These primers were used to amplify DNA made from the YACs DYR19–39 (76B1–2; B8–10), DYN26–35 (76A1–2; B2–3), DYN09–46 (76B8–11; D2–4), DYN09–31 (76C1–2; D5–8), DYN07–56 (76B5; B11) (Ajioka et al., 1991 Cai et al., 1994) or from wild-type Canton-S genomic DNA. All YACs were kindly provided by I. Duncan. Genomic DNA was prepared as described in Allada et al. (1998). The entire sequence of GM02625 was then determined using ABI PRISM sequencing. This clone was found to contain the entire coding sequence and is the same size as a cDNA isolated from a *Drosophila* head library (J. R., unpublished data). Sequence from the 3′ end of the clone was then used to design a unique primer (1572–1550) for reverse transcription of polyA$^+$ RNA made from *cyc*^{0} mutant flies and from a sibling control line as described previously (Rutila et al., 1996). The primer pairs corresponding to nucleotides (a) 40–68 + 1329–1306, (b) 1–21 + 304–285, (c) 239–258 + 793–772, (d) 578–596 + 1329–1306, and (e) 578–596 + 1452–1433 were used to amplify each cDNA, products were purified, and both strands sequenced from nested primers by ABI PRISM sequencing. Only one nucleotide change was identified between the *cyc*^{0} strain and a sibling strain obtained from the same mutagenesis. This change was identified from sequencing both strands of two different PCR products. The A to T transversion at nucleotide 586 changes a lysine codon (AAG) at amino acid 159 to a stop codon (UAG).

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GenBank Accession Number

The sequence of cycle has been submitted to GenBank under accession number AF065473. All nucleotide numbers refer to this sequence.