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Duper: A Mutation that Shortens Hamster Circadian Period

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Abstract Three animals born to homozygous *tau* mutant (τ_{ss}, "super short") Syrian hamsters showed extremely short free-running periods of locomotor activity (τ_{DD} of approximately 17.8 hours). Inbreeding produced 33 such "super duper" animals, which had a τ_{DD} of 18.09 ± 0.05 hours, which was shorter than that of τ_{ss} hamsters (20.66 ± 0.07 hours, $p < 0.001$). To test the hypothesis that a gene (*Duper*) is responsible for a 2-hour shortening of τ_{DD}, we backcrossed super duper hamsters to unrelated τ_{ss} animals. The F_1 pups uniformly had a τ_{DD} similar to that of τ_{ss} hamsters (19.89 ± 0.15 hours), but F_2 animals showed a 1:1 ratio of the 18- to 20-hour phenotypes. In contrast, the F_1 of a cross between super duper hamsters and τ_{ss} animals presumed heterozygous for *duper* showed a 1:1 ratio of 18- to 20-hour phenotypes, and inbreeding of the super duper F_1 offspring uniformly produced F_2 pups with extremely short τ_{DD} (17.86 ± 0.5 hours). We isolated the *duper* mutation on a wild-type background through crossing of super duper with wild-type animals. Restriction digests identified short-period F_2 pups that lack the mutant *CK1*ε allele, and these animals had a mean τ_{DD} of 23.11 ± 0.04 hours. τ_{DD} of duper hamsters born and raised in DD was significantly shorter than in hamsters raised in 14L:10D (21.92 ± 0.12 hours, $p < 0.0001$). τ_{DD} shortened twice as much in τ_s and τ_{ss} hamsters than in wild-type animals that were homozygous for *duper*, indicating the presence of epistatic interactions. Assortment of phenotypes in the F_2 generation fit the expected distribution for expression of *duper* as recessive ($\chi^2 = 6.41$, $p > 0.1$). Neither CK1ε nor CK1δ coding region base sequences differed between super duper and τ_{ss} hamsters. The growth rate of super duper mutants is similar to that of τ_{ss} animals but slightly but significantly reduced at particular postweaning time points. We conclude that *duper* represents a new mutation that substantially reduces τ_{DD} and has significant effects on physiology and metabolism.

Key words circadian, free-running rhythm, *tau* mutation, hamster, Period gene

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Period mutants have proven valuable not only in understanding the molecular mechanisms that generate circadian rhythms but also in establishing the role of such oscillations in photoperiodism, reproductive cycles, and other physiological functions. The first mammalian circadian mutation to be discovered was *tau* in Syrian hamsters (Ralph and Menaker, 1988). Because of the semidominant nature of this mutation, the heterozygous founder of this strain was observed to have a τ_{DD} of 22 hours. In its homozygous state (designated τ_{ss} and referred to colloquially as "super short"), this mutation results in a free-running period in constant darkness (τ_{DD}) of 20 hours. Lowrey et al. (2000) identified *tau* as an allele of *casein kinase 1*ε (*CK1*ε). The dramatic shortening of circadian period is due to a G-C transition that introduces a *BstAP1* restriction site and results in a change of amino acid residue 178 from arginine to cysteine. Subsequent work indicates that this change results in a gain of function, enhancing the activity of *CK1*ε to phosphorylate PERIOD proteins (Gallego et al., 2006; Eide et al., 2005; Meng et al., 2008). CK1δ, which is closely homologous to CK1ε, may also determine circadian period through regulation of the stability of clock proteins (Xu et al., 2007; Meng et al., 2008; Etchegaray et al., 2009; Lee et al., 2009; Walton et al., 2009). It has been proposed that accelerated clearance of PER2 is responsible for the shortening of circadian period in τ_{ss} hamsters (Dey et al., 2005; Meng et al., 2008).

In the course of experiments in our laboratory, we noticed an abnormally short τ_{DD} in 3 offspring of *tau* hamsters. We suspected that a mutation had spontaneously occurred on the τ_{ss} background, resulting in a phenotype we termed "super duper." In the present article, we describe results of studies undertaken to establish the dominance and inheritance pattern of this new mutation, which we term "*duper,*" to characterize its effects on circadian locomotor rhythms in DD and to establish by restriction digests and genetic sequencing that it is distinct from the *tau* mutation. In the companion article (Krug et al., 2011), we describe effects of the *duper* mutation on entrainment and phase resetting in response to light pulses.

MATERIALS AND METHODS

Animal Maintenance

Unless otherwise specified, hamsters were born and raised in 14L:10D. All had ad libitum access to food and water. During the light phase, white fluorescent bulbs provided approximately 400 lux at cage level. In order to assess circadian phenotype, locomotor activity was monitored in DD for a minimum of 10 days, during which animals were maintained individually in plastic tubs containing running wheels (17-cm diameter). Free-running period was calculated by linear regression using ClockLab software (Actimetrics, Evanston, IL) and confirmed by χ^2 periodogram analysis when appropriate. All procedures were approved by the animal care and use committee (IACUC) of the University of Massachusetts at Amherst and conform to all US federal animal welfare requirements.

Genetics and Crosses

Crosses were performed in order to assess dominance of the *duper* mutation on both *tau* mutant and wild-type backgrounds. Animals showing τ_{DD} of 18.2 hours or less were bred with *tau* mutant animals, and progeny were placed in running wheels in DD when they reached adulthood. These F_1 hamsters were returned to 14L:10D. Estrous cycles were monitored by vaginal smears (Orsini, 1961), and hamsters bred with other F_1 individuals showing the same super duper or *tau* phenotype. F_2 hamsters resulting from these crosses were similarly assessed for circadian period.

In order to isolate the *duper* mutation on a wild-type background, we first crossed super duper mutants with wild-type hamsters. When they reached young adulthood, F_1 hamsters resulting from this cross were placed in running wheels in DD in order to determine their circadian phenotype. Skin samples (ear clips) were enzymatically digested to obtain genomic DNA, and CK1ε was amplified as described by Lowrey et al. (2000). Restriction digestion was carried out using *BstAP1* in order to identify the *tau* locus, which results in a 137-bp cleavage product that can be visualized on a 3% MetaPhor gel (Cambrex Bio Science Inc, Rockland, ME). These hamsters were then returned to 14L:10D and inbred to produce an F_2 generation. These animals were also placed in DD as young adults to determine circadian phenotype, and genotype was assessed as for the parental generation. F_2 hamsters that lacked the *tau* mutation but whose τ_{DD} was below 23.2 hours were used for further breeding.

Duper hamsters in this generation were also crossed with τ_{ss} hamsters in order to produce offspring that were heterozygous for both *duper* and the *tau* alleles. We also assessed circadian rhythms of F_2 offspring of duper hamsters that were homozygous or

heterozygous for the *tau* (CK1ε) mutation. In addition, we backcrossed duper homozygotes that lacked the CK1ε mutation with wild-type hamsters in order to produce hamsters that were heterozygous for *duper* but bore no other circadian mutations. This allowed us to assess the dominance of the *duper* mutation.

Sequencing of Casein Kinase 1ε and 1δ

Total RNA was isolated from livers of wild-type, τ_{ss}, and super duper hamsters ($n = 5$, 3, and 7, respectively) using Ultraspec II isolation reagent (Biotecx Laboratories, Friendswood, TX) according to the supplier's directions. After DNase treatment (Ambion, Austin, TX), the complete coding regions of CK1ε and CK1δ were amplified by 1-step RT-PCR, cloned into pGEM-T easy (Promega, Fitchburg, WI) and sequenced (Genewiz Inc., South Plainfield, NJ). Primer sequences for CK1ε were adapted from Lowrey et al. (2000) (F: 5′-GCCCCGGCGAATCCTCTGGCATC-3′; R: 5′-TGAAGACACTAAGCAAACACTGGTC-3′). These primers extend through the end of coding in exon 9 and were enriched with nested primers, as previously described by Etchegaray et al. (2009). Midsequence primers were designed to span and cross the *tau* mutation site: F: 5′-GGCCAAGAAGTACCGTGATG-3′; R: 5′- AGGAGTAGTCGGGCTTGTCA-3′. As there was no sequence for hamster CK1δ in the database, we designed primers for nt 300 to 1601 (*Mus musculus* Csnk1(δ) variant 1: NM_139059.2, CDS nt 324-1571) and 300 to 1664 (variant 2: NM_027874.2, CDS nt 324-1553) from within exons 1 and 10. The sequence of the forward and reverse primers was 5′-GCAGTAGCGAGCCGCA-3′ and 5′-GTCTGCCCTTCACAGCAAAA-3′, respectively. The PCR product was further amplified using midsequence primers (F: 5′-TGGCATTGAACAATCTCGAA-3′; R: 5′-GCAAATTCAGAAGGATAGCC-3′). The product was sequenced and entered into Genbank (accession #GQ214551). Alignments were performed using BioLign alignment and multiple contig editor (Version 4.0.6.2, 2005; http://en.bio-soft.net/dna/BioLign.html).

Statistical Analyses

Free-running period was calculated by linear regression using ClockLab software (Actimetrics) and confirmed by χ^2 periodogram analysis when appropriate. Effects of genotype on free-running period were evaluated by analysis of variance (Super Anova, Abacus Concepts, Berkeley, CA). Student-Neuman-Keuls tests were used for post hoc comparisons, and the Student *t* test was performed for pair-wise tests. Correlation coefficients were generated using Microsoft Excel (Redmond, WA), and JMP statistical software (SAS Institute, Cary, NC) was used to calculate correlation coefficients between α, ρ, and τ and evaluate their statistical significance. The main effects of genotype and sex on postweaning body weight were evaluated using a repeated measures MANOVA (JMP statistical software, SAS Institute). Upon finding statistically significant interactions between genotype and age, we evaluated the differences in slopes of the body weight curves to determine effects of the *tau* and duper mutations alone and in combination on growth rates.

RESULTS

Origin of the Super Duper Strain

In the course of breeding τ_{ss} hamsters in 14L:10D for other experiments, we observed 2 hamsters (MK5 and MV1) with abnormally short τ_{DD} values (17.8 and 17.7 hours, respectively). Although these animals had different mothers, they were both offspring of the same male (MF1), whose τ_{DD} was 20.4 hours. We hypothesized that both MF1 and the mothers of MK5 and MV1 were heterozygous for a new mutation. Several attempts to breed MK5 were unsuccessful, but 2 of the offspring of one of his siblings had abnormally short τ_{DD} (17.5 and 17.7 hours). We were able to cross MV1 with these offspring of the sibling of MK5 to produce hamsters whose τ_{DD} values ranged between 17.8 and 18.7 hours. Because these animals arose spontaneously on a super short background, we referred to them as "super duper" hamsters. Among 33 hamsters produced from the first 2 generations of crosses in this line, we observed τ_{DD} of 18.09 ± 0.05 hours (mean ± SEM) upon transfer from 14L:10D (Fig. 1). This was significantly shorter than the free-running period of a group of not only 11 wild-type hamsters ($\tau_{DD} = 23.99 \pm 0.02$ hours) but also 27 τ_{ss} hamsters ($\tau_{DD} = 20.66 \pm 0.07$ hours) maintained in the same constant dark conditions ($p < 0.01$).

Vaginal smears of super duper females maintained in 14L:10D or 11L:10D revealed that most individuals sustain consecutive 4-day estrous cycles, although in some instances, positive smears occur at intervals of 5 days and more rarely at 3-day intervals. Female super duper hamsters maintained in DD often showed the "scalloping" pattern described in wild-type animals and attributed to fluctuating levels of ovarian hormones (Fitzgerald and Zucker, 1976; Morin et al., 1977)

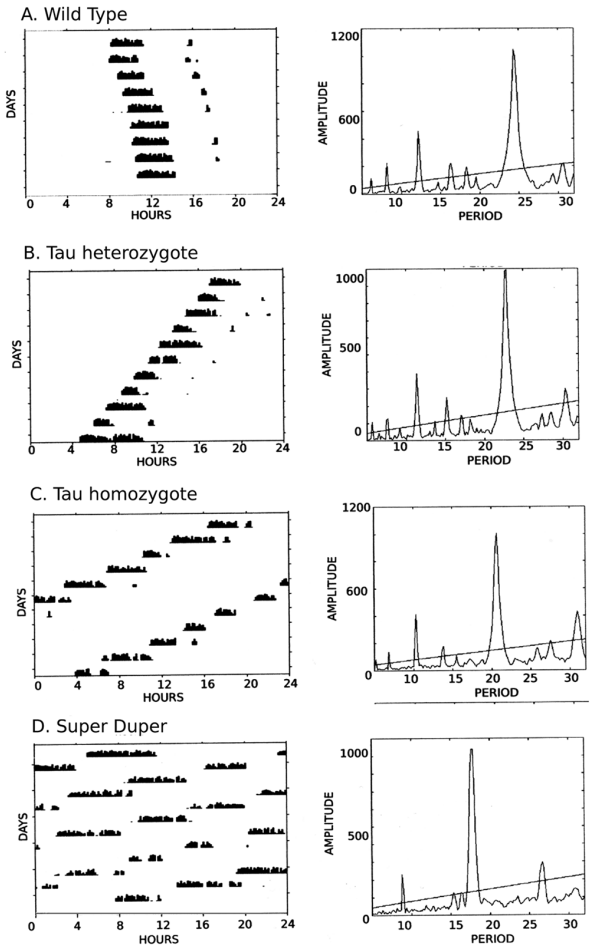

Figure 1. Actograms (left) and corresponding χ^2 periodograms (right) of representative (A) wild-type (τ_{DD} = 24.3 hours), (B) τ_s (τ_{DD} = 22.8 hours), (C) super short (τ_{DD} = 20.6 hours), and (D) super duper (τ_{DD} = 17.6 hours) Syrian hamsters allowed ad libitum access to running wheels in DD.

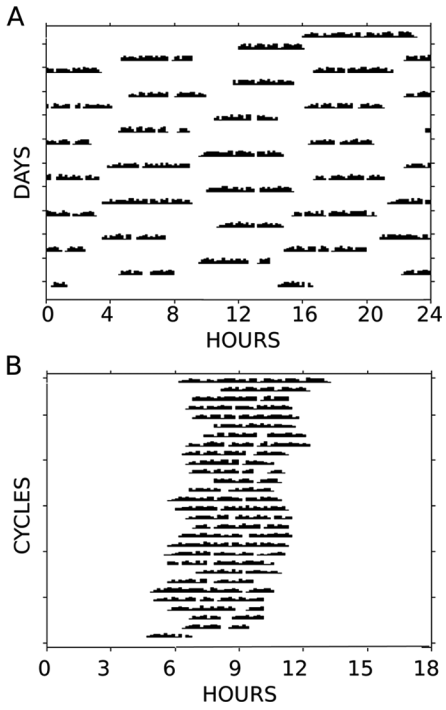

Figure 2. Locomotor activity record of a representative female super duper hamster in DD. The same data are plotted modulo 24 hours (top) and modulo 17.8 hours (bottom). Note "scalloping" reflecting periodic fluctuation of circadian onsets and α.

(Fig. 2). Although fecundity and litter size have not been systematically assessed in super duper hamsters, pups are born after a 16-day gestation as in wild-type and *tau* mutant hamsters. As expected, the slope of the curve fitted to the increase of body weight over time was lower in super duper and τ_{ss} than in wild-type and τ_s hamsters, and these animals reached a lower adult body weight. Although the rate of growth was similar between super duper and τ_{ss} hamsters, body weights of the former group were significantly lower at weeks 6 and 7 (*t* test, $p < 0.05$) (Suppl. Fig. S1).

Genetic Crosses

In order to determine whether super duper hamsters are homozygous for a recessive mutation, F_1 backcrosses were performed by breeding hamsters with $17.7 \leq \tau_{DD} \leq 18.3$ hours, with others showing the 20-hour τ_{ss} phenotype. The results varied between litters presumably because some hamsters with a τ_{DD} of 20 hours were heterozygous for, and others lacked, the mutant *duper* allele. In 3 cases, we crossed males showing the super duper phenotype (offspring of MV1 with $\tau_{DD} < 18$ hours) with offspring of siblings of MK5. Of the 16 hamsters produced in these litters, 7 showed a super duper phenotype, and 9 were of the super short phenotype. Three F_2 litters were produced by crosses among the super duper hamsters of the F_1 generation (Fig. 3A). As adults, these pups all had a super duper τ_{DD} (17.86 ± 0.05 hours, n = 23) (Fig. 3B). In another cross, we bred a τ_{ss} hamster unrelated to the super duper line (PN1, τ_{DD} = 20.2 hours) with a super duper female (PU1, τ_{DD} = 17.8 hours). All 6 offspring of this cross had short τ_{DD} in the lower range of periods, as reported by Lowrey et al. (2000) ($19.6 < \tau_{DD} < 20.6$; mean, 19.89 ± 0.15 hours) (Fig. 3C). The 25 F_2 hamsters produced by crosses within these litters were equally divided between super duper and super short phenotypes (Fig. 3D).

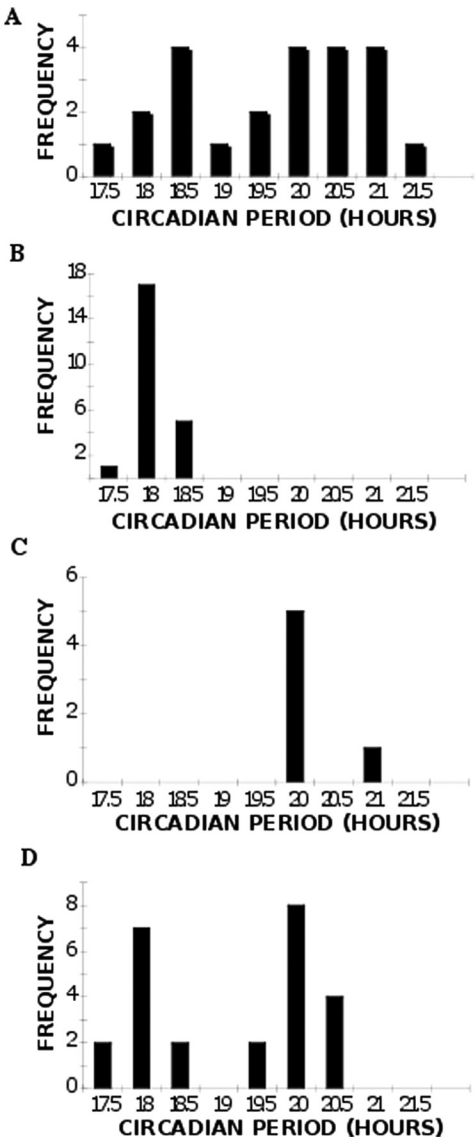

Figure 3. Number of animals (frequency) exhibiting free-running circadian periods (τ_{DD}) in the indicated histogram bins among offspring of crosses between super duper hamsters and animals of the super short phenotype. (A) Distribution of τ_{DD} in F_1 animals (7 litters) produced by crossing super duper hamsters with τ_{ss} hamsters (τ_{DD} = 20 hours) from the lineage that produced the first duper mutants and are presumed heterozygous for *duper*. (B) Distribution of circadian periods in F_2 progeny (3 litters) produced by inbreeding super duper animals of this cross. (C) Distribution of τ_{DD} in F_1 animals (1 litter) produced by crossing a super duper hamster (τ_{DD} = 18 hours) with an unrelated τ_{ss} animal that apparently lacked the *duper* allele. (D) Distribution of F_2 progeny of this cross (4 litters).

Isolation of the Duper Mutation on a Wild-Type Background

We first crossed super duper hamsters with wild types. The offspring of these matings had uniformly intermediate periods (τ_{DD} = 22.39 ± 0.05 hours, n = 16) (Table 1), and restriction digests confirmed that all were heterozygous for *tau*. These F_1 hamsters were next inbred to produce an F_2 generation of 101 hamsters, whose circadian periods ranged from 17.6 to 24.6 hours (Fig. 4).

Each parent could contribute 1 of 4 combinations of the wild-type (T, D) and mutant CK1ε (τ) and *duper* (d) alleles to their offspring (TD, Td, τD, or τd, respectively). If the *duper* allele is neither sex linked nor lethal, pups of 9 different genotypes are expected in classic Mendelian ratios (wild type, TTDD, 1/16; *duper* heterozygote, TTDd, 2/16; τ_s, TτDD, 2/16; double heterozygote, TτDd, 4/16; *duper* on τ_s background, Tτdd, 2/16; τ_{ss}, ττDD, 1/16; *duper* heterozygote on τ_{ss} background, ττDd, 2/16; *super duper*, ττdd, 1/16; and *duper* on wild-type background, TTdd, 1/16). Based on previous findings (Lowrey et al., 2000) and the results of the restriction digests (Fig. 5) we performed on tissue collected from hamsters in which we assessed free-running period, we expected the phenotype of the F_2 pups to fall into 5 nonoverlapping ranges. TTDD and TTDd animals are expected to have the longest free-running periods (τ_{DD} > 23.8 hours). The free-running periods of TτDD, TτDd, and TTdd hamsters that have overlapping ranges are expected to show 23.4 > τ_{DD} > 21.8. We anticipate the phenotype of ττDD and ττDd to be 21.0 > τ_{DD} > 19.8. Tτdd are expected to show 19.8 > τ_{DD} > 18.9, and ττdd hamsters are expected to have τ_{DD} < 18.5 hours. Empirically, we found the ratio of these phenotypes in the F_2 to be 16:51:15:17:2. A χ^2 analysis indicates that the distribution of periods does not differ from the pattern expected ($\chi^2 = 6.41$, $df = 4$, $0.2 < p < 0.1$).

Direct identification of the *duper* genotype was not possible because the allele is not yet identified. However, genotypic analysis for the *tau* allele was carried out, and the circadian period was in many cases shorter than would be expected on the basis of the CK1ε digest pattern alone. Significantly, 3 hamsters in the F_2 generation with τ_{DD} of approximately 22.3 hours were found by *BstpAP1* digest to completely lack the *tau* mutation (Fig. 5). Two litters produced by inbreeding of these offspring included 24 pups that carried only the wild-type CK1ε allele but whose τ_{DD} upon transfer from 14L:10D to constant darkness as young adults was between 22.7 and 23.4 hours (τ_{DD} = 23.12 ± 0.04 hours, mean ± SEM). In addition, 19 of the F_2 offspring had a τ_{DD} of less than 21 hours but were found by genotyping to be heterozygous for the *tau* mutation. Further crosses of τ_s heterozygotes with periods of 20 hours and below with each other and with *duper* animals lacking the *tau* allele allowed us to generate

Table 1. Characteristics of free-running circadian rhythms of wild-type and mutant hamsters maintained in constant darkness.

Genotype	τ_{DD}, hours	α, hours	Revolutions/ Cycle
Wild type	$23.97 \pm 0.02^{a,b,c,d,f,g}$ ($n = 15$)	$6.74 \pm 0.40^{a,c,e}$	$3170 \pm 351^{b,c,e}$
τ_s	$22.54 \pm 0.20^{b,e}$ ($n = 17$)	6.67 ± 0.53	$4611 \pm 633^{b,c,e}$
τ_{ss}	$20.40 \pm 0.08^{b,c,d,e}$ ($n = 17$)	5.61 ± 0.30^{e}	3611 ± 588
Duper (wt bkg)	23.12 ± 0.04^{e} ($n = 30$)	$6.80 \pm 0.24^{a,c}$	$6664 \pm 397^{a,b}$
Super duper (τ_{ss}/ homozygous duper)	$17.92 \pm 0.07^{b,c,d,e,f,g}$ ($n = 20$)	$4.92 \pm 0.26^{b,e}$	$2372 \pm 217^{a,b}$
Wild type/ heterozygous duper	24.16 ± 0.04^{a} ($n = 22$)	$7.90 \pm 0.247^{a,b,c,d}$	7818 ± 351^{b}
τ_s/heterozygous duper	$22.39 \pm 0.05^{b,e}$ ($n = 15$)	$5.58 \pm 0.33^{b,e}$	$7589 \pm 717^{a,b}$
τ_s/homozygous duper	$20.07 \pm 0.076^{b,c,d,e,f}$ ($n = 15$)	5.58 ± 0.25^{e}	$4319 \pm 461^{b,c}$

Data are presented as mean ± SEM. τ_{DD} = period of activity onsets; α = activity duration; wt bkg = wild-type background.
[a]$p < 0.05$ versus super duper; Student-Newman-Keuls test. [b]$p < 0.05$ versus duper. [c]$p < 0.05$ versus τ_s/heterozygous duper. [d]$p < 0.05$ versus τ_s. [e]$p < 0.05$ versus wild type/ heterozygous duper. [f]$p < 0.05$ versus τ_{ss}. [g]$p < 0.05$ versus τ_s/homozygous duper.

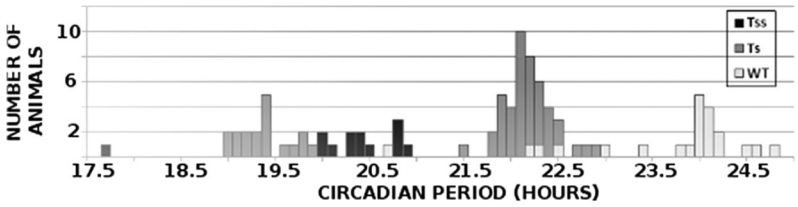

Figure 4. Distribution of circadian phenotypes of F_2 hamsters (second generation of offspring of super duper × wild-type cross). Genotype determined by amplification of CK1ε after *BstAP1* digest. Note that several hamsters lacking the *tau* mutation ("WT" genotype) showed a circadian period substantially below 24 hours. Inbreeding of these hamsters produced a line of duper hamsters on the wild-type background.

another 15 τ_s animals carrying *duper*. These results indicate that the *duper* mutation produces a 1-hour shortening of τ_{DD} when expressed on a wild-type background but a 2-hour shortening on either a *tau* heterozygote or homozygote background (Table 1).

Effect of Photoperiod of Gestation and Rearing on the Duper Phenotype

The foregoing experiments examined locomotor rhythms in animals that had been born and raised on a 14L:10D photoperiod. We considered the possibility that τ_{DD} of duper hamsters was influenced by an aftereffect of entrainment to 14L:10D by phase delays prior to assessment of free-running rhythms in DD (Krug et al., 2011). In order to evaluate such effects of the light cycle of gestation and rearing, we compared the τ_{DD} of 2 litters of *duper* parents that were born and raised in DD with that of animals of identical parentage that were born and raised in 14L:10D. As young adults, the dark-reared animals were placed in running wheels and found to have a locomotor period of 21.92 ± 0.12 hours ($n = 7$). Thus, rearing in DD resulted in a circadian period significantly shorter than that of duper animals reared in 14L:10D ($F_{1,26} = 9.68$, $p < 0.0001$). In contrast, α did not differ significantly between duper hamsters raised in DD and those raised in 14L:10D ($F = 1.22$, $p > 0.1$).

We were also concerned that the estimate of circadian period of super duper hamsters might be influenced by rearing in 14L:10D. To the extent that mutant animals entrain or relatively coordinate (Krug et al., 2011), the estimate of τ_{DD} could in part reflect an aftereffect of T. We therefore compared τ_{DD} between groups of super short hamsters born and raised in T21 (10L:11D, to which they reliably entrain) (Bittman, unpublished data) with those gestated and reared in T24 (14L:10D). τ_{DD} in these 2 groups of hamsters was 18.22 ± 0.06 hours ($n = 22$) and 17.99 ± 0.05 hours ($n = 40$), respectively. This comparison indicated that rearing in the longer T cycle actually produced a shorter τ_{DD} ($t_{2,61} = 2.94$, $p < 0.005$). One pair of super duper hamsters (NO4 and MV1) produced one litter on each of these T cycles. Pups born to these parents in T21 had a τ_{DD} of 18.23 ± 0.10 hours, while their siblings gestated and raised in T24 had a τ_{DD} of 17.73 ± 0.16 hours ($t_{2,10} = 2.65$, $p > 0.05$). Thus, τ_{DD} was no shorter in super duper hamsters raised in T21 than in those raised in T24.

Sequencing of CK1ε and CK1δ

In order to confirm that the duper trait arises from a distinctly different mutation than that which gives

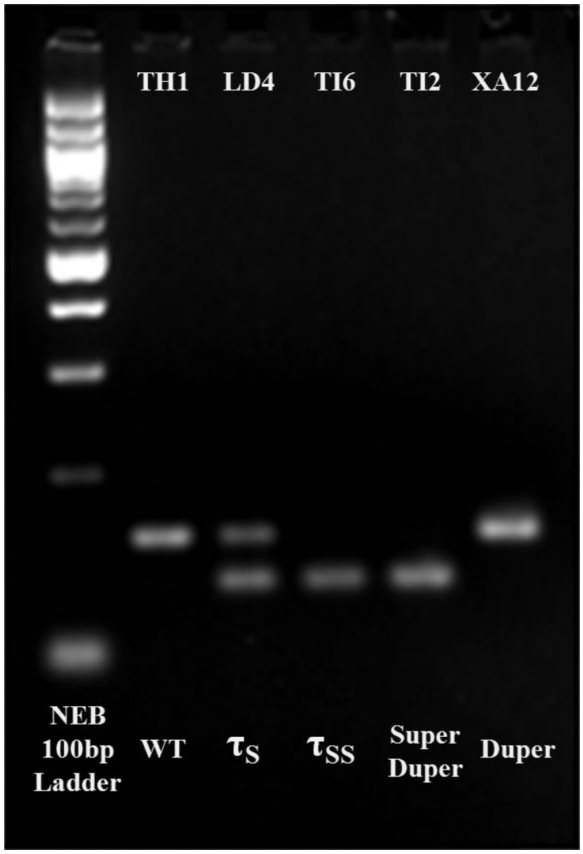

Figure 5. Representative 3% agarose gel depicting *BstAP1* digests of genomic DNA from a wild-type (TH1, τ_{DD} = 23.99 hours), a *tau* heterozygote (LD4, τ_{DD} = 22.71 hours), a super duper (TI2, τ_{DD} = 17.71 hours), and a duper (XA12, τ_{DD} = 22.95 hours) hamster. Genomic DNA was amplified, as described by Lowrey et al. (2000). A 100-bp DNA ladder is shown at the left for reference.

rise to the short period of the τ_{ss} strain in which it initially arose, or from a mutation in the coding region of the closely related CK1δ, we prepared and sequenced cDNA for these enzymes from liver samples of super duper animals. Neither sequence differed from that of super short hamsters (n = 4), nor did the CK1δ sequence in either the τ_{ss} (n = 3) or super dupers (n = 7) differ from that of the wild type (n = 5). In all cases, the CK1δ amplified from the hamster corresponded only to variant 2 of the mouse sequence (NM_027874.2: nt 300-1664).

DISCUSSION

We have identified a new mutation that arose spontaneously in *tau* mutant Syrian hamsters. When expressed on this background in the homozygous form, the duper mutation reduces the circadian period by about 2 hours. To our knowledge, the τ_{DD} of 18 hours shown by super duper hamsters is the shortest circadian period of locomotor activity so far recorded for a mammal. Although this period might be considered to be below the circadian range, it is likely that these free-running rhythms are generated by the same mechanism that underlies circadian oscillations of wild-type and τ_{ss} hamsters.

The effects of the *duper* and *tau* mutations on circadian period are similar. Thus, it was only in hamsters that were homozygous for both of these mutations that the circadian phenotype was clearly below the range previously described. We suspect that the *duper* mutation became evident only when homozygotes were produced through crosses of τ_{ss} animals that were heterozygous for this newly discovered allele. Results of the backcross experiments support this interpretation. Crossing super duper hamsters with τ_{ss} animals unrelated to the line in which the new mutation arose generated offspring whose τ_{DD} was in the lower range of the super short phenotype. The F_2 generation included super duper and super short animals in equal numbers. In contrast, when super duper hamsters were bred with more closely related animals likely to be carrying one copy of *duper*, F_1 hamsters with τ_{DD} of 18 and 20 hours were produced in a ratio of approximately 1:1. Most important, inbreeding of animals with τ_{DD} of 18 hours invariably resulted in offspring with a similar (super duper) period.

Animals that are wild type for *CK1*ε but heterozygous for *duper* show no shortening of period, indicating that in this condition, the mutation is recessive. Similarly, τ_{DD} of τ_s animals that are heterozygous for *duper* is comparable to that of *tau* heterozygotes that lack *duper* entirely. In contrast, τ_{DD} in τ_{ss} hamsters that are heterozygous for *duper* is about half an hour shorter than in *tau* homozygotes that lack *duper* ($p < 0.05$). This suggests that the degree to which duper is recessive depends upon the genetic background, although this requires further study with a larger number of animals. Furthermore, epistatic interactions are indicated by the finding that τ_{DD} shortens twice as much in the animals that are homozygous for duper and either heterozygous or homozygous for the *tau* mutation than in animals that are homozygous for *duper* and carry the wild-type *CK1*ε allele. The finding that 2 circadian mutations may have additive or multiplicative effects on circadian period is not unprecedented (Rothenfluh et al., 2000; Lakin-Thomas and Brody, 1985). When expressed as a percentage of τ_{DD}, the shortening of period in the super duper mutant is less than multiplicative but

more than additive. On the τ_s background, however, the effect of *duper* is close to multiplicative. It is important to note, however, that duper mutants entrain to 14L:10D and show an aftereffect that increases τ_{DD} (Krug et al., 2011). In contrast, τ_{ss} animals either do not entrain to 14L:10D (Ralph and Menaker, 1988) or do so erratically and with a markedly positive phase angle (Lowrey et al., 2000; Krug et al., 2011). Thus, the comparison of the effects of *tau* and *duper* on period, and assessment of their additive or multiplicative interactions, is complicated by the differences in aftereffects of prior conditions on τ_{DD}.

In addition to its effect to shorten the free-running period, the *duper* mutation alters the entrainment and phase-shifting properties of the circadian system (Krug et al., 2011). τ_{DD} was slightly but significantly longer in super duper hamsters raised in T21 than those reared in T24. Within offspring of the same parents, the photoperiod of gestation and rearing had no significant effect on τ_{DD} in super duper hamsters. This suggests that aftereffects of gestation and rearing in conventional photoperiods do not contribute in any substantial way to the reduction of τ_{DD} in super duper hamsters. If anything, exposure to T24 would be expected to lengthen τ_{DD} in such animals. A complete test of the influence of photoperiod of rearing would require exposure of super duper hamsters to T cycles shorter than 18 hours. A more thorough examination of entrainment and aftereffects during rearing and adulthood is presented in the companion article (Krug et al., 2011).

The identity of the mutated allele is unknown. Although our finding that the sequence of *CK1ε* is not altered in the duper mutants may be viewed as a negative result, it was critically important to establish that the mutation differs from that of the strain in which it arose. Our results indicate that it is unlikely that a second mutation in *CK1ε* has caused a further shortening of period by amplifying the effect of the arginine to cysteine conversion at residue 178. In light of the evidence that CK1δ is highly homologous to CK1ε, may phosphorylate clock proteins, and plays a major role in determining circadian period (Meng et al., 2008; Etchegaray et al., 2009; Lee et al., 2009; Walton et al., 2009), we also explored the possibility that a change in the CK1δ sequence may underlie the duper mutation. Our results do not support this hypothesis. In contrast to mice, we found evidence for the expression of only one variant of CK1δ in Syrian hamsters. While the significance of this finding is unclear, we found no evidence for a difference between super duper, τ_{ss}, and wild-type animals in the CK1δ sequence. We cannot rule out the possibility that the shortening of period in super duper animals results from a base change in either CK1 isoform outside the region that we amplified in the present experiments. It is also possible that a change in CK1 expression, perhaps arising from a change in the promoter region or a mutation altering autophosphorylation and/or the activity of another protein that acts upon CK1ε or δ, could account for the duper phenotype.

In addition to determining the genetic basis of the phenotype, it will be important to establish whether the effects of the *duper* mutation are restricted to locomotor activity cycles or whether *duper* has a general influence on cell-autonomous circadian rhythms in particular tissues or throughout the animal. The effects of the *duper* mutation on PRC amplitude, presented in the companion article (Krug et al., 2011), suggest that the protein product of *duper* may regulate expression of core components of the transcriptional-translational feedback loop (van der Horst et al., 1999; Bae et al., 2001). Such effects could include changes in posttranscriptional processes that regulate the stability, translocation, and nuclear residence time of the protein products of these genes (Tsuchiya et al., 2009; Tamaru et al., 2009; Maier, 2009; Hirota and Kay, 2008; Spengler and Antoch, 2009; Robles and Weitz, 2010; Eide et al., 2005; Partch et al., 2006; Cardone et al., 2005; Etchegaray et al., 2003; Ripperger and Schibler, 2006). Although it seems unlikely that *duper* primarily controls an output pathway, this possibility cannot be excluded. Mice deficient in D element-binding protein (DBP) show a significant shortening of τ_{DD} of locomotor activity (Lopez-Molina et al., 1997). Interestingly, super duper hamsters lack a circadian rhythm of *Dbp* expression in the kidney (Krug and Bittman, in preparation).

We anticipate that a mutation that causes a profound change in period should have significant physiological effects. Indeed, super duper hamsters showed retarded growth curves, amplifying the effect of the *tau* mutation on the rate of weight gain. This is consistent with the far-reaching influence of the circadian system on metabolism, health, and disease (Oklejewicz et al., 1997, 2001; Lucas et al., 2000). Nevertheless, the slope of the body weight curve was similar in super duper and τ_{ss} hamsters, indicating that growth rate is not a monotonic function of circadian period and that effects of mutations of the circadian system on metabolism may depend more upon their impact on entrainment than on τ_{DD} (Martino et al., 2008; Krug et al., 2011). Furthermore, these animals are fertile and show regular estrous cycles. The effects of the mutation on the LH

surge system, photoperiodism, and other aspects of physiology (Stirland et al., 1996; Loudon et al., 1998; Lucas et al., 1999) require further investigation.

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CONFLICT OF INTEREST STATEMENT

The author(s) have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

NOTE

Supplementary material for this article is available on the *Journal of Biological Rhythms* website at http://jbr.sagepub.com/supplemental.

REFERENCES

Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, and Weaver DR (2001) Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. Neuron 30:525-536.

Cardone L, Hirayama J, Giordano F, Tamaru T, Palvimo JJ, and Sassone-Corsi P (2005) Circadian clock control by SUMOylation of BMAL1. Science 309:1390-1394.

Dey J, Carr AJ, Cagampang FR, Semikhodskii AS, Loudon AS, Hastings MH, and Maywood ES (2005) The tau mutation in the Syrian hamster differentially reprograms the circadian clock in the SCN and peripheral tissues. J Biol Rhythms 20:99-110.

Eide EJ, Woolf MF, Kang H, Woolf P, Hurst W, Camacho F, Vielhaber EL, Giovanni A, and Virshup DM (2005) Control of mammalian circadian rhythm by CKIepsilon-regulated proteasome-mediated PER2 degradation. Mol Cell Biol 25:2795-2807.

Etchegaray JP, Lee C, Wade PA, and Reppert SM (2003) Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. Nature 421: 177-182.

Etchegaray JP, Machida KK, Noton E, Constance CM, Dallmann R, Di Napoli MN, DeBruyne JP, Lambert CM, Yu EA, Reppert SM, and Weaver DR (2009) Casein kinase 1 delta regulates the pace of the mammalian circadian clock. Mol Cell Biol 29:3853-3866.

Fitzgerald K and Zucker I (1976) Circadian organization of the estrous cycle of the golden hamster. Proc Natl Acad Sci U S A 73:2923-2927.

Gallego M, Eide EJ, Woolf MF, Virshup DM, and Forger DB (2006) An opposite role for tau in circadian rhythms revealed by mathematical modeling. Proc Natl Acad Sci U S A 103:10618-10623.

Hirota T, Lewis WG, Liu AC, Lee JW, Schultz PG, and Kay SA (2008) A chemical biology approach reveals period shortening of the mammalian circadian clock by specific inhibition of GSK-3beta. Proc Natl Acad Sci U S A 105:20746-20451.

Krug S, McKinley Brewer J, Bois AS, and Bittman EL (2011) Effects of the *Duper* mutation on circadian responses to light. J Biol Rhythms 26:293-304.

Lakin-Thomas PL and Brody S (1985) Circadian rhythms in Neurospora crassa: interactions between clock mutations. Genetics 109:49-66.

Lee H, Chen R, Lee Y, Yoo S, and Lee C (2009) Essential roles of CKIdelta and CKIepsilon in the mammalian circadian clock. Proc Natl Acad Sci U S A 106:21359-21364.

Lopez-Molina L, Conquet F, dubois-Dauphin M, and Schibler U (1997) The DBP gene is expressed according to a circadian rhythm in the suprachiasmatic nucleus and influences circadian behavior. EMBO J 16:6762-6771.

Loudon AS, Ihara N, and Menaker M (1998) Effects of a circadian mutation on seasonality in Syrian hamsters (*Mesocricetus auratus*). Proc Biol Sci 265:517-521.

Lowrey PL, Shimomura K, Antoch MP, Yamazaki S, Zemenides PD, Ralph MR, Menaker M, and Takahashi JS (2000) Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. Science 288:483-492.

Lucas RJ, Stirland JA, Darrow JM, Menaker M, and Loudon AS (1999) Free running circadian rhythms of melatonin, luteinizing hormone, and cortisol in Syrian hamsters bearing the circadian tau mutation. Endocrinology 140:758-764.

Lucas RJ, Stirland JA, Mohammad YN, and Loudon AS (2000) Postnatal growth rate and gonadal development in circadian tau mutant hamsters reared in constant dim red light. J Reprod Fertil 118:327-330.

Maier B, Wendt S, Vanselow JT, Wallach T, Reischl S, Oehmke S, Schlosser A, and Kramer A (2009) A large-scale functional RNAi screen reveals a role for CK2 in the mammalian circadian clock. Genes Dev. 23:708-718.

Martino TA, Oudit GY, Herzenberg AM, Tata N, Koletar MM, Kabir GM, Belsham DD, Backx PH, Ralph MR, and Sole MJ (2008) Circadian rhythm disorganization produces profound cardiovascular and renal disease in hamsters. Am J Physiol Regul Integr Comp Physiol 294: R1675-R1683.

Meng QJ, Logunova L, Maywood ES, Gallego M, Lebiecki J, Brown TM, Sladek M, Semikhodskii AS, Glossop NR, Piggins HD, et al. (2008) Setting clock speed in mammals:

the CK1 epsilon tau mutation in mice accelerates circadian pacemakers by selectively destabilizing PERIOD proteins. Neuron 58:78-88.

Morin LP, Fitzgerald KM, and Zucker I (1977) Estradiol shortens the period of hamster circadian rhythms. Science 196:305-307.

Oklejewicz M, Hut RA, Daan S, Loudon AS, and Stirland AJ (1997) Metabolic rate changes proportionally to circadian frequency in tau mutant Syrian hamsters. J Biol Rhythms 12:413-422.

Oklejewicz M, Pen I, Durieux GC, and Daan S (2001) Maternal and pup genotype contribution to growth in wild-type and tau mutant syrian hamsters. Behav Genet 31:383-391.

Orsini MW (1961) The external vaginal phenomena characterizing the stages of the estrous cycle, pregnancy, pseudopregnancy, lactation, and the anestrous hamster, *Mesocricetus auratus* Waterhouse. Proc Anim Care Panel 11:193-206.

Partch CL, Shields KF, Thompson CL, Selby CP, and Sancar A (2006) Posttranslational regulation of the mammalian circadian clock by cryptochrome and protein phosphatase 5. Proc Natl Acad Sci U S A 103:10467-10472.

Ralph MR and Menaker M (1988) A mutation of the circadian system in golden hamsters. Science 241:1225-1227.

Ripperger JA and Schibler U (2006) Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drives circadian dbp transcription and chromatin transitions. Nat Genet 38:369-374.

Robles MS, Boyault C, Knutti D, Padmanabhan K, and Weitz CJ (2010) Identification of RACK1 and protein kinase Calpha as integral components of the mammalian circadian clock. Science 327:463-466.

Rothenfluh A, Abodeely M, Price JL, and Young MW (2000) Isolation and analysis of six timeless alleles that cause short- or long-period circadian rhythms in Drosophila. Genetics 156:665-675.

Spengler ML, Kuropatwinski KK, Schumer M, and Antoch MP (2009) A serine cluster mediates BMAL1-dependent CLOCK phosphorylation and degradation. Cell Cycle 8:4138-4146.

Stirland JA, Mohammad YN, and Loudon AS (1996) A mutation of the circadian timing system (tau gene) in the seasonally breeding Syrian hamster alters the reproductive response to photoperiod change. Proc Biol Sci 263:345-350.

Tamaru T, Hirayama J, Isojima Y, Nagai K, Norioka S, Takamatsu K, and Sassone-Corsi P (2009) CK2alpha phosphorylates BMAL1 to regulate the mammalian clock. Nat Struct Mol Biol 16:446-448.

Tsuchiya Y, Akashi M, Matsuda M, Goto K, Miyata Y, Node K, and Nishida E (2009) Involvement of the protein kinase CK2 in the regulation of mammalian circadian rhythms. Sci Signal 2:ra26.

van der Horst GT, Muijtjens M, Kobayashi K, Takano R, Kanno S, Takao M, de Wit J, Verkerk A, Eker AP, van Leenen D, et al. (1999) Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. Nature 398:627-630.

Walton KM, Fisher K, Rubitski D, Marconi M, Meng QJ, Sladek M, Adams J, Bass M, Chadrashekaran R, Butler T, et al. (2009) Selective inhibition of casein kinase 1 epsilon minimally alters circadian clock period. J Pharmacol Exp Ther 330:430-439.

Xu Y, Toh KL, Jones CR, Shin JY, Fu YH, and Ptacek LJ (2007) Modeling of a human circadian mutation yields insights into clock regulation by PER2. Cell 128:59-70.