A Length Polymorphism in the Circadian Clock Gene *Per3* is Linked to Delayed Sleep Phase Syndrome and Extreme Diurnal Preference

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Study Objectives: To investigate the link between extreme diurnal preference, delayed sleep phase syndrome, and a length polymorphism in *Per3*.

Design: Subjects were genotyped using polymerase chain reaction.

Patients or Participants: Subjects with defined diurnal preference as determined by the Horne-Östberg questionnaire and patients with delayed sleep phase syndrome.

Measurements and Results: The *Per3* polymorphism correlated significantly with extreme diurnal preference, the longer allele associating with morningness and the shorter allele with eveningness. The shorter allele was strongly associated with the delayed sleep phase syndrome patients, 75% of whom were homozygous.

Conclusion: The length of the *Per3* repeat region identifies a potential genetic marker for extreme diurnal preference.

Key Words: Circadian rhythms; phosphorylation; polymorphism (genetics); protein kinases; sleep disorder, circadian rhythm

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INTRODUCTION

SLEEP TIMING AND STRUCTURE ARE STRONGLY INFLUENCED BY THE CIRCADIAN SYSTEM,[1] which anticipates day length and generates daily rhythms from a master pacemaker in the suprachiasmatic nuclei.[2] Every day, environmental photic time cues are processed via retinal input pathways to synchronize (entrain) the circadian pacemaker to the 24-hour day. In the absence of external time cues, the free-running endogenous circadian period τ is expressed. Diurnal preference, as determined by the Horne-Östberg (HO) questionnaire[3], a validated quantitative tool, has been shown to correlate with τ.[4] The relatively rare conditions known as advanced and delayed sleep phase syndromes (ASPS/DSPS) have been described as pathologic extremes of diurnal preference and may be linked to extremely short or long τ, respectively.[5]

The accepted model for the molecular machinery that generates circadian rhythms involves a number of clock genes and their products.[6] The *Period* (*Per*) gene family is a central component in this mechanism, providing negative auto-feedback on its own expression. *Per* transcripts and PER proteins oscillate with period lengths correlated to the observed τ.[7] Phosphorylation targets PER for degradation, imposing a rate-limiting step on the amount of PER protein available for dimerization and subsequent nuclear translocation. A mutation in *Per2* has been reported to associate with ASPS, potentially by disrupting a target site for phosphorylation by casein kinase 1 (CK1) ε.

Here, we report a novel link between a length polymorphism in *Per3* and diurnal preference in humans. Homozygous *Per3* knockout mice display a free-running τ 30 minutes shorter than the wildtype.[8] Five *Per3* polymorphisms have been reported in a Japanese population, occurring in four haplotypes.[9] One of these haplotypes was reported to be more frequent in DSPS subjects, although the association between the five polymorphisms within this haplotype and the disorder were not determined. Taking a different approach, we focused specifically on a length-polymorphic repeat region composed of either 4 or 5 units, which is described, but not specifically analyzed, in the previous paper. The prevalence of this polymorphism was studied both in subjects with extreme diurnal preference and in DSPS patients.

METHODS

Out of 484 volunteers who completed the Horne-Östberg questionnaire and donated buccal DNA samples, the 7% of subjects with the highest (morning preference) and lowest (evening preference) HO scores were selected, together with a control group of equal size with an intermediate HO score, as described in a previous report.[10] Blood samples were also collected from 16 unrelated patients (8 males, 8 females, aged 16-27 years) suffering from intrinsic DSPS, also described earlier.[10] Informed consent was obtained from all subjects after explanation of the nature of the study. The study was granted approval by the institutional Advisory Committee on Ethics and followed the tenets of the Declaration of Helsinki. Genotyping was performed using polymerase chain reaction with the primers described by Ebisawa et al [9] using the ProofSprinter polymerase mixture (Hybaid, Ashford, Kent) and the following amplification conditions: 94°C for 3 minutes, then 38 cycles of 94°C for 45 seconds, 58° for 45 seconds, and 72° for 1 minute. Agarose gel electrophoresis was used to identify whether individuals were heterozygous or homozygous for either of the *Per3* repeat alleles.

RESULTS

Figure 1 shows the frequency of the 4- and 5-repeat alleles in groups with extreme evening and extreme morning preference, as well as the intermediate group. A significant trend was observed between the three groups (χ^2 test for trend, P=0.030), with the frequency of the 5-repeat allele significantly higher in the morning-preference (5-repeat: 0.42, 4-repeat: 0.58) compared to the evening-preference group (5-repeat: 0.24, 4-repeat: 0.76; Fisher's Exact Test, P=0.047, odds ratio=2.205). In the DSPS patient group, the frequency of the 4-repeat allele was significantly higher (5-repeat: 0.12, 4-repeat: 0.88), compared to the total

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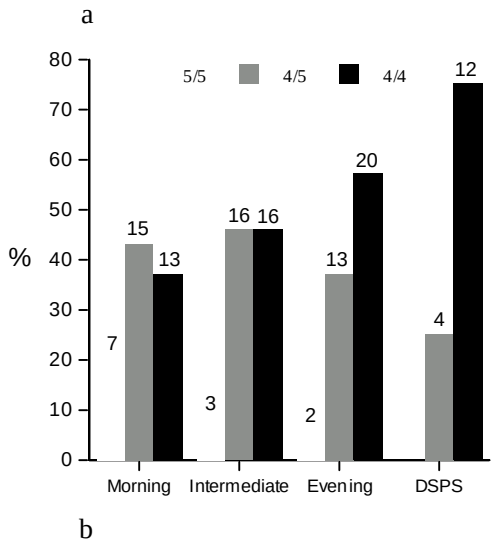
control population (all 105 selected individuals; 5-repeat: 0.32, 4-repeat: 0.68) (Fisher's Exact Test, P=0.0224, odds ratio=3.352). No 5/5 homozygotes were found in the DSPS group, and 75% were homozygous for the 4-repeat.

DISCUSSION

This is the first reported correlation between a polymorphism in a clock gene coding region and extreme diurnal preference in humans, including DSPS. The earlier publication by Ebisawa and coworkers[9] does not report this correlation in their material. This may be a reflection of their study being based on carrier rather than allele frequencies, ethnic differences, or both. Our findings provide some insights into the potential function of *Per3*. CKIε phosphorylates all three PER proteins, regulating their stability and nuclear translocation.[11] Each of the 4- or 5-repeat sequences in PER3 contain potential CK1ε phosphorylation motifs clustered in a similar arrangement to those found in PER2 (Figure 2).[12] Of the amino acid residues in the repeat region, 32% are identifiable as potential substrates for phosphorylation, as compared to 10% in the protein as a whole. Phosphorylation by CK1ε is enhanced by prephosphorylation of a lead serine or threonine residue in the recognition motif. In PER2, a mutation in the first of a cluster of tandemly arranged CK1ε recognition motifs has been hypothesized to reduce the chain of local phosphorylation leading to a more stabilized protein product and associated ASPS. In PER3, the decreased number of amino acid residues available as phosphorylation substrates in the shorter variant would predict a functional polymorphism in phosphorylation-dependent effects. Because these sites are also arranged in a tandem array, the mechanism may be very similar to that proposed in PER2. The identification of a robust link between this polymorphism and extreme diurnal preference in humans indicates a precise way in which differential PER3 phosphorylation may contribute to the phenotypic difference. This finding identifies a priority area for future studies and potential pharmacologic intervention. It also identifies a potential genetic marker for extreme diurnal preference, which may prove clinically useful in the differential diagnosis of DSPS. Because of the limited number of DSPS sufferers analyzed in this study, our finding should be confirmed in a larger cohort. It will also be of interest to investigate its prevalence in other sleep disorders, as well as its biochemical effects, including potential differences in phosphorylation. The ability to tolerate night shift work, time zone transitions, and artificial time cues in a 24-hour society is likely to depend upon the presence of specific clock gene variants, such as the one reported here.



Per3 allele frequencies

	4 repeat	5 repeat
Morning	0.58	0.42
Intermediate	0.68	0.32
Evening	0.76	0.24
Combined	0.68	0.32
DSPS	0.88	0.12

Figure 1—(a) Percentage of subjects with each of the *Per3* repeat genotypes in the morning-, intermediate- and evening-preference subject groups. Numbers are indicated above the bar for each genotype. (b) Frequency of alleles (N = 70) in morning-, intermediate- and evening-preference groups, in the 3 groups combined (N = 210), and in the delayed sleep phase syndrome (DSPS) group (N = 32).

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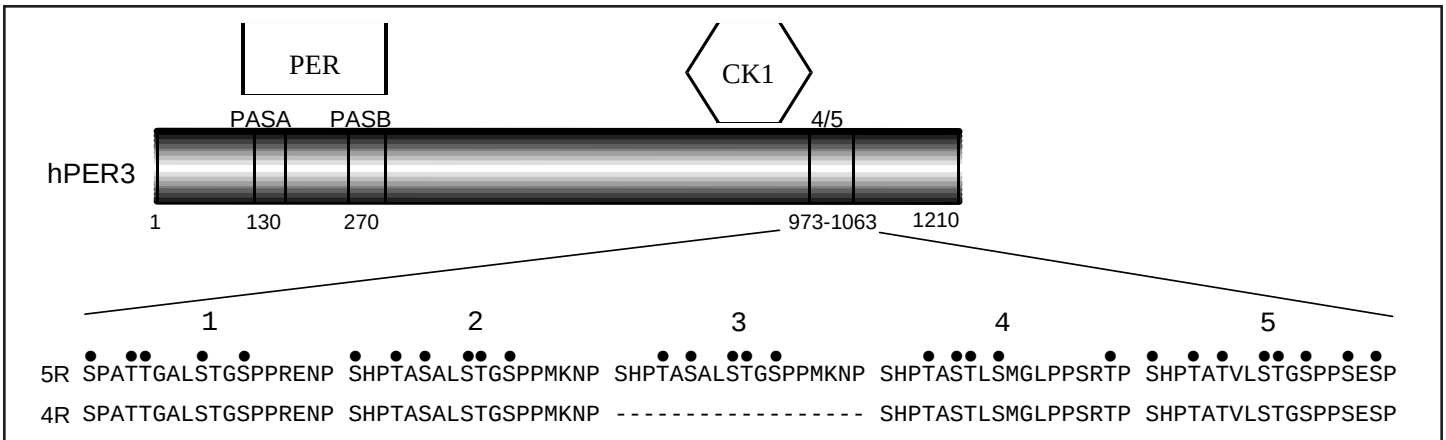

Figure 2—Schematic diagram of the human PER3 protein. Binding to other PER molecules occurs at the PAS A and B domains. The 5- and 4-repeat region amino acid sequences are shown expanded below the sequence (5R and 4R, respectively). The repeats are numbered 1-5 and the one that is missing in the 4-repeat allele is indicated by dashes. Predicted targets for casein kinase 1 (CK1) ε phosphorylation are indicated above by filled circles (the consensus CK1ε motif is Sp/Tp-$X_{1\text{-}3}$-S/T, where the lead serine or threonine must be prephosphorylated, X is 1-3 nonspecific spacing residues, and the target serine or threonine is underlined).

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