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Virtually all organisms express circadian oscillations of physiology, biochemistry, or behavior that reflect their need to anticipate the 24 hour periodicity of night and day. Genetic heterogeneity underlies many phenotypic variations observed in circadian rhythmicity. In order to identify genetic loci that underlie this complex behavior, we have carried out a genome-wide complex trait analysis in (C57BL/6J x BALB/cJ)F_2 hybrid mice. We have characterized variation in five circadian phenotypes: free-running circadian period, phase angle of entrainment, amplitude of the circadian rhythm, circadian activity level, and dissociation of rhythmicity. Quantitative trait locus analysis of these phenotypes has led to the identification of 13 loci having significant effects on this behavior. Although single gene mutations can affect circadian rhythms, the analysis of interstrain variants demonstrates that significant genetic complexity underlies this behavior. Importantly, the large majority of the loci that we have detected by these methods map to locations that differ from the nine known clock genes, indicating the presence of additional clock-relevant genes in the mammalian circadian system. These data demonstrate the analytical value of quantitative trait locus analysis in understanding complex phenotypes, and point to promising approaches for genetic analysis of such phenotypes in other mammals, including humans.

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Report:

Original Abstract from Proposal: Virtually all organisms generate self-sustained oscillations of physiology, biochemistry, or behavior that reflect their need to anticipate the 24-hour periodicity of night and day. Among many animals, the most obvious of these rhythms is a daily cycle of activity and rest. Considerable evidence indicates that this cycle is controlled by a program of oscillating macromolecular synthesis; elucidating the molecular mechanism of this program is the central goal of our laboratory. Isolating the genes that direct the circadian program in invertebrates has been the key to our present mechanistic understanding of circadian behavior in this group, and a similar strategy will likely be necessary if we are ever to do the same in mammals. Accordingly, our laboratory has highlighted the role of one gene in circadian activity, *Clock*, through a mutagenesis screen of the mouse. The expression of the gene's mutant phenotype, however, can be modified by the genetic background in which we place it, indicating that the *Clock* gene interacts with other genetic factors in the circadian program. An effort to dissect this interaction genetically with quantitative trait locus analysis promises to provide further insight into the molecular and biochemical mechanisms of the mammalian circadian system. To this end we propose to construct a two-generation intercross between C57B/6J mice homozygous for the *Clock* mutation and wild type mice of the CAST/Ei strain; to map in F_2 animals heterozygous for the *Clock* mutation genetic markers linked to variable expression of *Clock's* activity phenotype; and finally, to introgress each newly identified locus into a separate congenic strain, free from co-segregating factors that may modify its expression. Once isolated, each locus's contribution to circadian function may be dissected rigorously with the tools of both classical genetics and molecular biology. At the same time, however, many of the technical developments that have made such genetic dissections a productive force in the mouse, have, when combined with innovations in experimental design and statistical analysis produced a like revolution in the field of human genetics. Increasingly, human genetic analysis of complex traits proceeds simultaneously with genetic dissections in the organisms that model them. We propose, therefore, in parallel with our analysis of the mouse, a complementary genetic dissection of a robust circadian phenotype in man - morningness versus eveningness. The proposed study aims to address the apparent complexity of this circadian behavior with a non-parametric allele sharing analysis of sibling pairs discordant for extremes of diurnal preference, as assessed with multiple measures. To this end, we propose to characterize in a genetically homogenous population a cohort of sibling pairs discordant for extreme morning and evening activity preference; to identify within this cohort microsatellite markers whose inheritance correlates with the extreme expression of the phenotype, and finally, to initiate an association analysis of candidate genes that will isolate the susceptibility alleles more discretely. The results from the proposed experiments promise to provide insight into the genetic basis of normative circadian behavior. Such information will ultimately permit the structural characterization of gene products, essential for targeted chemical interventions into the ubiquitous circadian disruptions

produced by shift work and rapid travel across time zones, as well as the circadian pathophysiology implicated in disorders of sleep, affect, and endocrinology.

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Summary of this Project

This project was originally approved for funding for a three-year period to study quantitative trait loci that regulate circadian rhythms in mice and to study diurnal preference in human subjects and use statistical genetics methods to find genes associated with this trait. Originally the proposal had 5 specific aims, which were reduced in scope because funding was terminated during the second year of the award (after 18 months at the original funding amount and for 6 months at a reduced rate).

Due to the reduced duration and amount of funding, the project was modified as follows.

1. The first two aims of the original proposal on quantitative trait loci analysis in the mouse were completed. This work has been submitted as an article to *Genome Research* and has been accepted for publication. The abstract (above) describes the results of this paper. (K. Shimomura, S.S. Low-Zeddies, D.P. King, T.D.L. Steeves, A. Whiteley, J. Kushla, P.D. Zemenides, A. Lin, M.H. Vitaterna, G.A. Churchill and J.S. Takahashi. Genome-Wide Epistatic Interaction Analysis Reveals Complex Genetic Determinants of Circadian Behavior in Mice. *Genome Research* (accepted).

2. Work on the last three aims of the original project on human rhythms was terminated. Progress had been made on all three of these aims and one study was completed and published (Katzenberg, D., T. Young, L. Finn, L. Lin, D.P. King, J.S. Takahashi and E. Mignot. 1998. A *CLOCK* polymorphism associated with human diurnal preferences. *Sleep*. 21:569-576). There were not sufficient funds or time to continue these studies.

Introduction

All living organisms on Earth exist in an environment that varies regularly with a frequency of 24 hours. The individual organism sees a daily cycle not only of abiotic parameters such as light and temperature, but also in all aspects of the biotic sphere which it inhabits. There has been strong evolutionary pressure for animals to develop genetic programs which temporally optimize their physiology and behavior on a daily basis, such as the cycle of rest and activity. Just as strictly as a species is organized in space, so also is its temporal organization precisely regulated. Functional control over overt circadian (about 24 hour) rhythms and their phasing relative to the environment delineates a critical temporal niche for each organism within its own ecosystem. The endogenous source of this potent control system is what we refer to as its circadian “clock”.

Several experimental observations are key to our further consideration of genetic influences on circadian rhythmicity. The following reflect properties that are thought of as intrinsic to the core clock mechanism. The first is that animals continue to express precise physiological and behavioral rhythms in an environment devoid of external temporal cues. The period of the

rhythm under these conditions, also referred to as the "free-running" period, is close to but usually not exactly 24 hours; period length is a species-dependent characteristic and is normally consistent over an animal's lifetime i.e. genetically determined. Secondly, a necessary feature of a pacemaker is the ability to integrate pertinent environmental information, principally light, to appropriately align its period and phase with the external oscillation. These phase-response characteristics of an organism's circadian pacemaker, in conjunction with period length, determine how it entrains to a light/dark cycle. Light alters the phase of the rhythm differentially depending on the phase at which it interacts with the ongoing circadian cycle. The phase is delayed by light during the early part of the clock's subjective night, and advanced by light during the late night. Light exposure at points during the day phase of the clock does not change its phase. A final universal feature of circadian clocks, which only rarely has relevance to homeotherms such as mammals, is the capacity to compensate for temperature effects on the rate of the biochemical processes underlying timekeeping. Evidence continues to mount suggesting that the core circadian oscillator mechanism is fundamentally similar in all organisms at a molecular, and, to some extent, even a genetic level.

In addition to the central pacemaker, or clock, the circadian timing system includes input and output signalling pathways. Input to the central oscillator transduces environmental information, such as light, as well as feedback about the state of the organism itself, such as whether the animal is awake or asleep. Output signals are hooked up to temporally regulate a wide range of physiological functions, many of which sustain their own entrainable oscillations. The details of these pathways diverge considerably across the animal kingdom, a consequence of species-specific evolutionary tailoring.

Quantitative Trait Locus (QTL) Analysis of Circadian Behavior

Although single genes can have major effects even on complex behavioral regulation, such overt organismal behavior will always emerge from interactions between multiple genetic and epigenetic factors. The challenge which we now face is to define more of the elements in the pathway to circadian rhythmicity, even in cases where their phenotypic effects may be somewhat subtle.

Quantitative trait locus (QTL) analysis, implemented in our laboratory, has proven capable of identifying multiple loci involved in circadian behavioral differences between strains of mice. Genetic heterogeneity underlies many phenotypic variations observed in circadian rhythmicity. In order to identify genetic loci that underlie this complex behavior, we have carried out a genome-wide complex trait analysis in (C57BL/6J x BALB/cJ)F_2 hybrid mice. We have characterized variation in five circadian phenotypes: free-running circadian period, phase angle of entrainment, amplitude of the circadian rhythm, circadian activity level, and dissociation of rhythmicity. Quantitative trait locus analysis of these phenotypes has led to the identification of 13 loci having significant effects on this behavior. Although single gene mutations can affect circadian rhythms, the analysis of interstrain variants demonstrates that significant genetic complexity underlies this behavior. Importantly, the large majority of the loci that we have

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Polymorphism in the Human *CLOCK* Locus

One of the central goals of circadian biology is to identify and elucidate the molecular components of the mammalian circadian pacemaker. Beyond providing insights into normative circadian physiology, this approach may facilitate the genetic analysis of conditions associated with dysfunction of the circadian system. Previously, we described the identification and molecular isolation of the *Clock* gene in the mouse (Vitaterna et al., 1994; Antoch et al., 1997; King et al., 1997), its interaction with the BMAL1 protein, and the role of this complex as an activator of transcription in the circadian pacemaker (Gekakis et al.,, 1998). We have extended our analysis of *Clock* to humans (Steeves et al. 1999). We have shown that human *CLOCK* is highly conserved with its mouse ortholog, and that its multiple transcripts are widely but variably expressed in the human brain and body. We have determined the exon structure and chromosomal location of the *CLOCK* gene and reported biallelic variations within it that will facilitate the genetic analysis of a number of human traits and conditions.

The discovery of a human gene intimately involved in the regulation of the circadian pacemaker presents new opportunities for the genetic analysis of a number of human traits and conditions associated with dysfunction of the circadian system. Association analysis with alleles of known genes is a powerful means of identifying the genetic determinants of complex traits and conditions (Risch and Merikangas, 1996). The identification of SNPs within *CLOCK* has provided a means of determining its allelic frequency within a selected population and so fulfills a necessary prerequisite for any analysis of its phenotypic effects. Although the DNA sequence variations we have described in *CLOCK* produce no obvious structural modifications in its protein, mutations outside the gene's coding sequence could nonetheless change its expression or alter the stability of its message. Alternately, any polymorphism that defined a *CLOCK* allele could also be only one sequence variation in linkage disequilibrium with others, as yet unidentified but more functionally significant.

At least one of these conditions appears to apply to the sequence variation (3111C / 3111T) we have described in the 3' UTR of *CLOCK*. We have reported (Katzenburg et al., 1998) the gene frequency of the 3111C and 3111T alleles (0.27 and 0.73 respectively) in a population of European ancestry, as well as a highly significant (p=0.02) association of the 3111C allele with the diurnal preferences of evening "types". The distinction between morning and evening types is a well-documented circadian phenomenon (Horne and Ostberg, 1976). It refers principally to a tendency among individuals to prefer specific times of day for mental and physical activity, often extending to include an advanced or delayed phase of activity onset relative to the solar day - i.e. early to bed, early to rise vs. late to bed, late to rise. However it is also phenotypic difference with numerous physiological correlates and wide ranging implications for health and performance. The association of the 3111C allele with evening

preference is the first functional evidence of a known gene contributing to variation in the circadian behavior of humans. The effect is independent of age, sex, or geographic origin, and is thus unlikely to have been produced by population stratification. The result is of additional interest, however, because it suggests that the function of the *CLOCK* gene in humans is truly orthologous and by extension that the gene itself is a candidate locus for genetic analyses of human circadian disorders.

References

Antoch, M.P., E.-J. Song, A.-M. Chang, M.H. Vitaterna, Y. Zhao, L.D. Wilsbacher, A.M. Sangoram, D.P. King, L.H. Pinto and J.S. Takahashi. 1997. Functional identification of the mouse circadian *Clock* gene by transgenic BAC rescue. *Cell* 89:655-667.

Gekakis, N., D. Staknis, H.B. Nguyen, F.C. Davis, L.D. Wilsbacher, D.P. King, J.S. Takahashi and C.J. Weitz. 1998. Role of the CLOCK protein in the mammalian circadian mechanism. *Science* 280:1564-1569.

Horne, J.A., and Ostberg, O. 1976. A self-assessment questionnaire to determine morningess-eveningness in human circadian rhythms. *Int. J. Chronobiol.* **4:** 97-110.

Katzenberg, D., Young, T., Finn, L., Lin, L, King, D.P., Takahashi, J.S., and Mignot, E. 1998. A *CLOCK* polymorphism associated with human diurnal preference. *Sleep* **21**: 569-576.

King, D.P., Y. Zhao, A.M. Sangoram, L.D. Wilsbacher, M. Tanaka, M.P. Antoch, T.D.L. Steeves, M.H. Vitaterna, J.M. Kornhauser, P.L. Lowrey, F.W. Turek and J.S. Takahashi. 1997. Positional cloning of the mouse circadian *Clock* gene. *Cell* 89:641-653.

Risch, N., and Merikangas, K. 1996. The future of genetic studies of complex human diseases. *Science* **273:** 1516-1517.

Shimomura, K., S.S. Low-Zeddies, D.P. King, T.D.L. Steeves, A. Whiteley, J. Kushla, P.D. Zemenides, A. Lin, M.H. Vitaterna, G.A. Churchill and J.S. Takahashi. Genome-wide epistatic interaction analysis reveals complex genetic determinants of circadian behavior in mice. *Genome Research* (accepted).

Steeves, T.D.L., D.P. King, Y. Zhao, A.M. Sangoram, F. Du, A.M. Bowcock, R.Y. Moore and J.S. Takahashi. 1999. Molecular cloning and characterization of the human *CLOCK* gene: Expression in the suprachiasmatic nucleus. *Genomics* 57:189-200

Vitaterna, M.H., D.P. King, A.-M. Chang, J.M. Kornhauser, P.L. Lowrey, J.D. McDonald, W.F. Dove, L.H. Pinto, F.W. Turek and J.S. Takahashi. 1994. Mutagenesis and mapping of a mouse gene, *Clock*, essential for circadian behavior. *Science* 264:719-725.