Transcriptional Profiling of mRNA Expression in the Mouse Distal Colon

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Background & Aims: **Intestinal epithelial cells and the myenteric plexus of the mouse gastrointestinal tract contain a circadian clock–based intrinsic timekeeping system. Because disruption of the biological clock has been associated with increased susceptibility to colon cancer and gastrointestinal symptoms, we aimed to identify rhythmically expressed genes in the mouse distal colon.** ***Methods:*** **Microarray analysis was used to identify genes that were rhythmically expressed over a 24-hour light/dark cycle. The transcripts were then classified according to expression pattern, function, and association with physiologic and pathophysiologic processes of the colon.** ***Results:*** **A circadian gene expression pattern was detected in approximately 3.7% of distal colonic genes. A large percentage of these genes were involved in cell signaling, differentiation, and proliferation and cell death. Of all the rhythmically expressed genes in the mouse colon, approximately 7% (64/906) have been associated with colorectal cancer formation (eg, B-cell leukemia/lymphoma-2 [Bcl2]) and 1.8% (18/906) with various colonic functions such as motility and secretion (eg, vasoactive intestinal polypeptide, cystic fibrosis transmembrane conductance regulator).** ***Conclusions:*** **A subset of genes in the murine colon follows a rhythmic expression pattern. These findings may have significant implications for colonic physiology and pathophysiology.**

Circadian rhythms are cyclic changes in physiologic parameters that occur over approximately 24 hours. The gastrointestinal tract exhibits several circadian biological rhythms. For example, gastric acid secretion, gastric emptying, digestive enzyme expression and activity along the crypt-villus axis, and response to certain drugs used in the treatment of patients with colon cancer vary substantially with the time of day.[1,2] Colonic motility follows a rhythm as well. Healthy people have bowel movements during the day, frequently following awakening or following a meal but rarely during the night.

A central pacemaker is located in the hypothalamic suprachiasmatic nucleus of the brain, which receives direct photic input via the retinohypothalamic tract. This central pacemaker or clock communicates with peripheral tissues via neuronal and humoral pathways by either driving rhythmic activity or, more likely, entraining peripheral oscillators, controlling the expression of a subset of tissue-specific processes and genes, thereby regulating organ-specific physiologic functions. It has been estimated that approximately 8%–10% of genes in peripheral organs are expressed in a rhythmic manner, suggesting that they are either directly or indirectly controlled by clock genes.

The molecular basis for biological rhythms is believed to be regulated by a set of so-called "clock genes" and their products within the suprachiasmatic nucleus as well as peripheral tissues. Clock genes are a group of genes that participate in an interlocked transcription-translation feedback loop, in which the "positive elements" (CLOCK and brain and muscle Arnt-like protein 1 [BMAL1]) form heterodimers and enter the nucleus. There, they bind to the promoter regions of the "negative elements" (*Period* genes [*Per*] and *Cryptochrome* genes [*Cry*]) and activate their transcription. The PER and CRY proteins slowly accumulate as heterodimers and feed back to inhibit CLOCK-BMAL1–dependent transcription, thereby closing the central loop.[3] A second interlocking loop involving the transcription of retinoic acid orphan-like receptor a (*Rora*) and *Reverbα*, which are also activated by CLOCK-BMAL1, amplifies the central loop by regulation of *Bmal1* transcription. The 2 interlocking loops are believed to generate the approximately 24-hour period of the molecular oscillator.[4]

In addition, these clock genes and their products can directly or indirectly regulate the transcription of a subset of tissue-specific genes,[3] affecting the output pathway(s) of the molecular clock. Previous gene array studies have suggested that approximately 5%–15% of genes in

Abbreviations used in this paper: CFTR, cystic fibrosis transmembrane conductance regulator; nNOS, neuronal nitric oxide synthase; PCR, polymerase chain reaction; ZT, Zeitgeber time.

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peripheral organs follow a rhythmic expression profile.[5] We have recently shown that clock genes are rhythmically expressed in the mouse gastrointestinal tract and are capable of shifting their phase of expression in response to changes in the feeding cycle.[6] Within the gastrointestinal tract, clock genes are expressed predominantly in the epithelial cells and the myenteric plexus, suggesting a possible role for clock genes in the coordination of cell proliferation, differentiation, and motility.[6] In addition, clock genes may drive the rhythmic expression of the sodium-hydrogen exchanger NHE_3 in the rat colon, suggesting a possible role for rhythmic regulation of salt absorption as well.[7]

Several clinical observations suggest a role for clock genes in the pathogenesis of gastrointestinal symptoms and disease. First, disruption of our cyclic environment secondary to shift work, time zone traveling, or space flights is associated with gastrointestinal symptoms such as abdominal discomfort, constipation, or diarrhea.[8–12] Second, irritable bowel syndrome is more common in nurses participating in shift work.[13] Finally, the gastrointestinal clock may play an important role in the pathogenesis of colon cancer. Data from the Nurses' Health Study showed that women who worked at least 3 nights per month for 15 or more years had a significantly greater risk of developing colorectal cancer when compared with women who never worked rotating night shifts.[14] On the basis of such studies, the International Agency for Research on Cancer, the cancer arm of the World Health Organization, considers overnight shift work a "probable carcinogen."[15]

Based on these observations, we hypothesized that a subset of genes in the gastrointestinal tract is transcriptionally regulated by the products of clock genes. These genes are likely to be involved in the regulation of rhythmic gastrointestinal events such as motility, cell differentiation, and proliferation. This study therefore aimed to determine temporal patterns of gene expression in the mouse distal colon. This profiling of gene expression was used to (1) determine the extent to which the transcriptome was rhythmically expressed in the distal colon and (2) characterize the functional distribution of rhythmically expressed genes within metabolic and signaling pathways relevant to gastrointestinal physiology.

Materials and Methods

Animals

Mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Male C57BL/6J mice were used for all experiments. Mice were 8–12 weeks of age at the onset of the experiment with body weights of 20–25 g. Experimental protocols involving animals were approved by the institutional animal care and use committee in accordance with the guidelines provided by the National Institutes of Health.

Time-of-Day Experiments

Mice were maintained for 2 weeks on a 12-hour light/dark cycle (lights on at 7 AM, lights off at 7 PM) before all experiments. Throughout this report, time is indicated using Zeitgeber time (ZT) as the indicator for the phase of the rhythm, whereby ZT0 refers to the time that lights went on (7 AM) and ZT12 refers to the time that lights went off (7 AM). Samples were collected every 4 hours starting at ZT1 with n = 3 mice per time point for all experiments. For the reference experiment, mice had ad libitum access to food and were kept under a strict 12-hour light/dark cycle. For a rhythm to be considered circadian, it has to persist in noncycling environmental conditions. Therefore, for the clock genes and a subset of colonic genes, we assessed rhythmicity in the absence of light or feeding. To remove the effect of light, mice were released into constant darkness for a total of 48 hours with ad libitum access to food. To remove the effect of feeding, mice were released into constant darkness for a total of 24 hours while fasting for solids but with ad libitum access to water. Under constant darkness, circadian time was referenced to the previous ZT such that the beginning of the subjective day was labeled ZT0 and the beginning of the subjective night was labeled ZT12.

Total RNA Extraction

The distal colon was identified following laparotomy and resected approximately 0.5 cm above the anus. Total RNA was isolated by modified guanidinium thiocyanate/phenol/chloroform extraction method and was treated with deoxyribonuclease I (Promega, Madison, WI) at 37°C for 30 minutes. RNA samples were quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and qualified by analysis on an RNA Nano Chip using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc, Santa Clara, CA).

Affymetrix GeneChip Analysis

Gene expression analysis was completed by the Molecular Genomics Core at the University of Texas Medical Branch using Affymetrix GeneChips mouse 430 2.0 (Affymetrix, Santa Clara, CA) according to protocols described in the Affymetrix GeneChip Expression Analysis Technical Manual. In brief, first-strand complementary DNA was synthesized from 15 μg of total RNA using an oligo(dT) primer encoding a bacteriophage T7 RNA polymerase promoter (SuperScript II Reverse Transcriptase; Invitrogen, Carlsbad, CA). Samples were prepared with varying concentrations of prokaryotic genes *dap*, *thr*, *lys*, *phe* (poly (A) controls that serve to address technical adequacy of the labeling and transcription process) and *bio B*, *bio C*, *bio D*, and *cre* (to address array sensitivity). Following second-strand synthesis, in vitro transcription was performed using T7 RNA polymerase in the presence of biotin-labeled nucleotides. Hybridization of the arrays