Functional Genomics of Sleep and Circadian Rhythm
Invited Review: Regulation of mammalian circadian clock genes

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Albrecht, Urs. Invited Review: Regulation of mammalian circadian clock genes. J Appl Physiol 92: 1348–1355, 2002; 10.1152/japplphysiol.00759.2001.—The circadian clock is a self-sustaining oscillator that has a period of ~24 h and controls many physiological and behavioral systems. This clock can synchronize itself to changing environmental conditions to optimize an organism's performance. The underlying circadian rhythms are generated by periodic activation of transcription by a set of clock genes. Besides their own regulation, clock genes can influence biochemical processes by modulating specific genes of biochemical pathways. Developments in the last few years using genetics and molecular biological tools have led to a new understanding of the molecular basis of the circadian clock in mammals. In this mini-review, I will summarize these advances that have led us to begin understanding the mammalian circadian clock at the molecular level.

clock gene mutations; oscillators; resetting

LIFE ON EARTH IS UNDER THE influence of the daily changes of night and day. No other environmental factor has influenced evolution in such a steady rhythm. Life that depends on energy source light “internalizes” its periodic availability in the form of the circadian clock. Circadian clocks regulate a diversity of activities in nature, such as the sleep-wake cycle, migration behavior in birds, and seasonal reproduction (22). Circadian rhythms are defined as oscillations that display a cycle with a period length of about 24 h, hence the term circadian from the Latin circa dies, which translates into “about one day.”

Konopka and Benzer (37) provided the first evidence that a single gene can have an impact on circadian rhythms by identifying mutations in the fruit fly Drosophila melanogaster that affected the circadian clock. They called the locus in which the mutations occurred period because the period length of circadian rhythmicity in these flies was affected. The corresponding molecular defect was identified in a single gene named period (per) according to the locus (10). In the years that followed, a number of additional circadian clock genes have been identified in Drosophila. These genes include timeless (tim), clock (clk), cycle (cyc), double-time (dbt), cryptochrome (cry), and vrille (vri) (5, 13, 36, 48, 59). Mutations in these genes alter the period length or lead to a loss of circadian rhythmicity. On the molecular level, it is thought that expression of per is driven by a heterodimer of CLK and CYC (59). CLK/CYC heterodimers bind to the E box present in the per promoter and thereby activate per transcription (20). Transcripts exit the nucleus and are translated in the cytoplasm of the cell. Subsequently, the PER protein is phosphorylated by a casein kinase I (CKI) encoded by the dbt gene (56). Phosphorylation affects PER stability and thereby influences the amount of PER that can be transported into the nucleus on interaction with TIM (17, 25, 61). In the nucleus, PER/TIM complexes block the activation of per transcription by interfering with the activity of CLK/CYC heterodimers. This negative feedback loop thus regulates per expression in Drosophila.

For most of the Drosophila circadian clock genes, orthologs have been identified in mammals, highlighting a general conservation in the clock mechanism between insects and mammals. In particular, three mammalian Period genes (mPer1, mPer2, and mPer3),
two Cryptochrome genes (mCry1 and mCry2), as well as Clock, Bmal1 (ortholog to cyc), and CKIε (ortholog to dbt) have been identified (3, 35, 36, 45, 69, 71, 84). Interestingly, the mammalian homolog for Drosophila tim, mTim, is more similar to a second Drosophila tim (tim2) (12), which has no function in the clock of the fruit fly. In mammals, it plays a role in kidney development (39), rather than in the circadian clock (28). More evidence is accumulating that, despite the similarity of the amino acid sequence of clock components between Drosophila and mammals, regulation of the mammalian clock is different from that described in Drosophila (6, 23, 82).

In mammals, clock genes are expressed in the suprachiasmatic nuclei (SCN), which are thought to be the pacemaker of the circadian clock [see review by Herzog and Schwartz in this issue (29a)]. Many of the clock genes are expressed in an oscillating manner on the transcriptional and on the translational level (Table 1). The different phases of oscillating gene expression nurture the idea that these phase differences are functionally significant for the clock (18, 50).

Interestingly, clock gene expression is not restricted to the SCN and can be observed in most tissues (3, 69). Oscillating gene expression is maintained in peripheral organs (81) and even in cell culture (8). This indicates that circadian rhythmicity is not cell type specific and is a property of individual cells.

### MUTATIONS IN MAMMALIAN CLOCK GENES

To begin to decipher the molecular mechanism of the mammalian circadian clock, mutant mice and hamsters that display aberrant circadian wheel running behavior have been investigated (35, 41, 57, 76). In addition, targeted mutations and deletions of candidate circadian clock genes have led to a better understanding of the function of clock genes in the mouse. If each of these clock genes would be essential for a single cycle in the clock, a null mutation in each of them would probably result in immediate arrhythmicity. As we will see below, this is not the case and it is reasonable to assume that there is partial redundancy of function among some subsets of clock gene products.

Loss or mutation of specific clock genes can lead to altered period length (Cry1, Cry2, Per1, Per3, CKIε) and slow (Clock, Per2) or immediate loss (Bmal1) of circadian rhythmicity under constant conditions (Table 2). Cry1/Cry2 double mutant animals (74, 77) and Per1/Per2 double mutant animals (6, 82) lose circadian rhythmicity immediately under constant conditions, confirming the importance of these genes in the clock mechanism. However, Per3 seems to be dispensable for the clock because deletion of Per3 has only a mild effect on circadian wheel running activity (63), and Per1/Per3 and Per2/Per3 double mutants display the phenotype of Per1 or Per2 mutants, respectively (6). The array of phenotypes observed after the disruption of the main molecular clock components suggests that not all clock gene products are equally important for the maintenance of circadian rhythmicity.

The only nonredundant gene in the clock so far is Bmal1 (Mop3). Deletion of this gene leads to immediate arrhythmicity in constant darkness. On the molecular level, there is no expression of Per1 and Per2 in the SCN, which indicates that Bmal1 positively regulates Per gene expression (14) (Fig. 1). Given the phenotype of the dominant-negative Clock mutation, the effects of Bmal1 on Per gene expression are consistent with an activator role of the CLOCK/BMAL1 transcription factor complex (26, 35).

Mice lacking both Cry1 and Cry2 are behaviorally arrhythmic (74, 77) and show constant noncycling expression of the Per1 and Per2 genes in the SCN and peripheral tissues (53). This indicates that the Cry genes act as negative regulators of Per gene expression (Fig. 1). Mice missing either the Cry1 or the Cry2 gene are rhythmic but display altered period lengths. Cry1<sup>−/−</sup> mice have a period shorter than 24 h, whereas the period of Cry2<sup>−/−</sup> mice is longer than 24 h (Table 2). In constant darkness, the clocks of these mice run faster or slower, respectively, supporting the notion that the clock consists of two opposite-acting oscillators (18, 55) (Fig. 1).

### Table 1. Time of maximal expression of mammalian clock genes in the suprachiasmatic nuclei of mice and rats

<table>
<thead>
<tr>
<th>Gene</th>
<th>CT or ZT, h</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPer1</td>
<td>4–6</td>
<td>23, 53, 65, 66, 69, 71</td>
</tr>
<tr>
<td>mPer2</td>
<td>9–12</td>
<td>3, 23, 53, 70</td>
</tr>
<tr>
<td>mPer3</td>
<td>4–6</td>
<td>70, 84</td>
</tr>
<tr>
<td>mCry1</td>
<td>8–12</td>
<td>45, 53</td>
</tr>
<tr>
<td>mCry2</td>
<td>12</td>
<td>53</td>
</tr>
<tr>
<td>Bmal1 (Mop3)</td>
<td>18</td>
<td>30, 64</td>
</tr>
</tbody>
</table>

CT, circadian time; ZT, zeitgeber time.

### Table 2. Mammalian circadian clock genes and the effect of mutations on period length in constant darkness and on the corresponding protein

<table>
<thead>
<tr>
<th>Gene Affected</th>
<th>Period Length in Constant Darkness, h</th>
<th>Protein</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmal1</td>
<td>No rhythm</td>
<td>Probably no functional protein Arg&lt;sup&gt;178&lt;/sup&gt; to Cys&lt;sup&gt;178&lt;/sup&gt; mutation</td>
<td>14, 41, 57</td>
</tr>
<tr>
<td>Cktε (hamster)</td>
<td>20–22</td>
<td>Deletion of amino acids 514–564</td>
<td>35, 76</td>
</tr>
<tr>
<td>Clock</td>
<td>27.26 ± 0.22 (loses rhythm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cry1</td>
<td>22.51 ± 0.06</td>
<td>No protein</td>
<td>74, 77</td>
</tr>
<tr>
<td>Cry2</td>
<td>24.63 ± 0.06</td>
<td>No protein</td>
<td>72, 74</td>
</tr>
<tr>
<td>Per1</td>
<td>21.6–23.8</td>
<td>No protein</td>
<td>16, 82</td>
</tr>
<tr>
<td>Per2</td>
<td>22.6 ± 0.3 (loses rhythm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Per3</td>
<td>23.27 ± 0.18 (loses rhythm)</td>
<td></td>
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</tr>
</tbody>
</table>

Mutation in Cktε is a spontaneous mutation identified in the hamster. All other mutations are in the mouse.

J Appl Physiol • VOL 92 • MARCH 2002 • www.jap.org
Mice with targeted deletions in Per1 and Per2 are behaviorally arrhythmic compared with the Cry1/Cry2 mice (6, 82), which demonstrates the necessity of these genes in the clock mechanism. A mutation in Per2 alone leads to gradual loss of circadian locomotor activity and to a reduced expression of the clock genes in the cell. Accumulation would lead to an equilibrium in transcriptional activation of clock genes and thus to a loss of rhythmicity in gene expression. One gene product that is thought to regulate clock genes posttranslationally is CK1ε. This gene codes for a kinase that is involved in phosphorylation of Per genes (36). A mutation in the CK1ε gene of hamsters, the so-called tau mutation, leads to a very short period length (Table 2 and Refs. 41, 57), demonstrating the importance of posttranslational regulation of clock components.

IN VITRO EXPERIMENTS

Transcriptional assays using cell culture systems have been applied to study the interaction of clock components in vitro. PER1 can modestly inhibit transcription induced by the CLOCK/BMAL1 complex (32, 62), which indicates that PER1 can act as a negative regulator of CLOCK/BMAL1-driven transcription (Fig. 1). A reduction or loss of PER2 protein in the nucleus leads to a shift in Bmal1 transcription (64) and sets the synchronized oscillating expression of clock components out of phase, which leads either to an unstable period length (82) or eventually to a loss of circadian rhythmicity (6) in Per1 mutant mice. Under constant light conditions, the period length of Per1 mutants is longer than 24 h, whereas for Per2 mutants it is shorter and no loss of rhythmicity is observed (S. Steinlechner and U. Albrecht, unpublished observations). This lends further support to the view that the clock consists of two opposite-acting oscillators (18).

Posttranslational regulation of clock components is essential to prevent accumulation of their proteins in the cell. Accumulation would lead to an equilibrium in transcriptional activation of clock genes and thus to a loss of rhythmicity in gene expression. One gene product that is thought to regulate clock genes posttranslationally is CK1ε. This gene codes for a kinase that is involved in phosphorylation of Per genes (36). A mutation in the CK1ε gene of hamsters, the so-called tau mutation, leads to a very short period length (Table 2 and Refs. 41, 57), demonstrating the importance of posttranslational regulation of clock components.

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Several lines of evidence suggest that PER and CRY proteins interact with themselves or each other, thereby forming stabilized complexes that influence nuclear transport or transcriptional regulation of clock genes (38, 79). Two-hybrid studies in yeast have shown that each of the three PER proteins can interact with itself, with the other two PERs, as well as with each of
the two CRY proteins (29, 85). Coimmunoprecipitation has demonstrated association of PER proteins with each other and with CRY proteins in SCN tissue (23). These findings indicate that complex protein-protein interactions occur. They seem to be regulated by post-translational modifications determining stability and nuclear entry of PER proteins (15, 34, 75). Subcellular localization of the clock components as well as the timely synchronized presence of specific components will determine which complexes of clock proteins are functionally relevant in vivo. In the future, it will be important to search for quantitative information about kinetics of reactions in which clock gene products participate and to know more about the dynamic interactions between different clock components.

SYNCHRONIZATION OF THE CLOCK TO THE ENVIRONMENT

The circadian clock runs with a period that is not exactly 24 h. Therefore, the clock needs to be reset periodically to ensure synchronization of the organism’s physiology to the environmental light-dark cycle. The value of the entrainment of the clock by environmental cues is that the organism’s endogenous phase becomes a reliable predictor of solar time. The dominant regulator of this entrainment is nocturnal light (54). Information about nocturnal light is transmitted in mammals by glutamate, which is released from retinal projections to the clock in the SCN. Activation of ionotropic glutamate receptors mediates Ca\(^{2+}\) influx (21) and ultimately leads to target gene activation. Because the Per1 and Per2 genes display a strong but differential light responsiveness, they could be target genes of the light transduction pathway (3, 23, 65, 66, 70). Mutations in the Per1 and Per2 genes show that they are required for normal photic resetting of the mammalian clock. Per1 mutants do not advance and Per2 mutants do not delay the clock normally (4). The Cry genes have also been implicated in behavioral responses to light (72). However, mice mutant in Cry1/ Cry2 retain the ability to have Per gene expression induced by light, suggesting that the Cry genes are dispensable for light-induced phase shifts (53).

The rapid activation of Per mRNA synthesis by light is probably due to phosphorylation of the transcription factor CREB (27, 47) and activation of mitogen-activated protein (MAP) kinases (1, 9, 47, 51). Because CREB and MAP kinase phosphorylation can be triggered by several cellular events, Per gene expression can also be synchronized to the clock by nonphotic cues (31, 43, 68). Synchronization of peripheral clocks by nonphotic cues is particularly important in mammals because light has no direct access to tissues such as the kidney or the liver. Although there are SCN efferents to peripheral tissues (reviewed in Ref. 11), serum inducibility of clock genes in rat-1 fibroblasts (8) suggests that blood-borne factors must stimulate signal transduction pathways that influence the molecular oscillators in cells of peripheral tissues. It has been shown that glucocorticoids, which are hormones secreted into the blood stream in daily cycles, can reset the circadian time by changing Per gene expression in peripheral tissues without affecting the central pacemaker in the SCN (7). In contrast to light, which activates Per gene expression only at night, glucocorticoid signaling can induce Per gene expression at all times of the day (7). This responsiveness at all times is expected from slave oscillators present in the periphery that are synchronized by the master pacemaker in the SCN.

In the vasculature, it has been shown that retinoic acid can phase shift Per2 mRNA rhythmicity in vivo and in serum-induced smooth muscle cells in vitro. Retinoic acid binds to its receptors RAR\(\alpha\) and RRX\(\alpha\), which interact with CLOCK/BMAL1 and MOP4/ BMAL1-mediated transcriptional activation of clock gene expression in smooth muscle vascular cells, providing a molecular mechanism for hormonal control of clock gene expression (44). The human Per1 promoter has also been shown to be activated by interleukin-6 in a manner additive to the cAMP pathway and independent of CREB phosphorylation and MAP kinase activation (46). The finding that interferon-\(\alpha\) can disrupt the rhythm of locomotor activity, body temperature, and clock gene expression in mice (52) reinforces the importance of blood-born factors on the circadian system.

TARGET GENES OF THE CIRCADIAN CLOCK

Many aspects of physiology and behavior are governed by the clock. It acts on neural and endocrine pathways to regulate individual circadian rhythms. This enables adaptation to daily and seasonal environment and enhances efficiency by separating anabolic and catabolic processes in time. However, the molecular connections between the clock mechanism and physiological circadian rhythms are poorly understood. Examination of the transcriptional regulation of the arginine vasopressin gene (AVP) revealed an involvement of the CLOCK/BMAL1 transcription complex (32). Mice mutant in the Per2 gene show abolished rhythmic expression of the AVP gene in the SCN, demonstrating circadian control of AVP gene expression in the SCN (2).

D-element binding protein (DBP) is a liver-enriched transcription factor that is expressed with a circadian period (78). It has been shown that this gene is regulated by CLOCK/BMAL1, indicating that clock genes can regulate rhythmic transcription of clock output regulators (58). In turn, DBP can bind to the promoter of the Per1 gene, thereby positively influencing Per1 gene transcription (80). This reflects the ability of the clock to react to its own target genes, which is expected to be an indispensable characteristic for sensing the physiological state of the organism. It might explain why restricted feeding can shift the period of the circadian clock in peripheral tissues such as liver and kidney without affecting the phase of the central pacemaker in the SCN (19).

A mutation in DBP shortens the period length slightly to 23.26 ± 0.08 h (40) and affects circadian...
sleep consolidation and rhythmic electroencephalogram activity (24). Sleep homeostasis is also altered in Clock mutant mice (49), and it remains to be seen whether other components of the circadian clock can alter sleeping behavior.

Analysis of differential gene expression in Per1 and Per2 mutant mice has revealed that several biochemical pathways are controlled by the clock (82). There are different classes of clock-controlled genes: 1) genes that are under the control of Per1, 2) genes that are under the control of Per2, and 3) genes that are dependent on both Per1 and Per2 genes (82). One of the clock-controlled genes identified is CRBP1, a gene that encodes a protein in vitamin A homeostasis. As described above, retinoic acid, a vitamin A precursor, can regulate clock gene expression in the vasculature (44). Therefore, it seems that the availability of vitamin A and the regulation of the circadian clock are interlocked, indicating that clock-controlled biochemical pathways can feed back to the clock. Another biosynthetic pathway that is under circadian control is heme synthesis. The circadian expression patterns of the genes for aminolaevulinate synthase 1 and 2, key enzymes in heme synthesis, are disturbed in Per mutant mice. This might lead to an altered temporal pattern in heme biosynthesis, which might be the root of abnormalities in these mice.

Given the fact that Per genes can respond to common signaling pathways (1, 9, 47, 51), it is very likely that clock-controlled pathways will feed back directly or indirectly to the clock. In line with this notion is the finding that cellular metabolism can regulate the activity of the clock. The heterodimerizations of transcription factors MOP4 (NPAS2) and BMAL1 that switch on target genes involved in circadian oscillations are regulated in vitro by the ratio between reduced and oxidized forms of the respiratory chain electron carriers NAD and NADP, which fluctuate according to the metabolic state of the cell (60). In this context, it is attractive to speculate that the electrons from NADH might be transferred to FAD associated with CRY from where the electrons would be transported to a heme cofactor that can bind to MOP4 and regulate binding of MOP4 to DNA. This would close the regulatory loop of heme synthesis and clock regulation.

WORKING MODEL OF THE MAMMALIAN CLOCK MECHANISM IN THE SCN

Expression analysis in conjunction with genetic findings described in the sections above lead to the hypothetical model of the circadian clock proposed by Daan et al. (18). A refined version of this model is shown in Fig. 1, which depicts an SCN neuron. In cells of the peripheral organs, some regulatory mechanisms, especially those concerning the feedback of clock controlled genes (ccg), are possibly cell type specific (44) and are therefore not included here.

The CLOCK and BMAL1 proteins dimerize and form a complex that can bind to the E boxes in the promoters of the Per1 and Per2 genes and activate their transcription. This probably happens in association with additional unknown factors. The PER1 and PER2 proteins become phosphorylated in the cytoplasm presumably by CK1e. This influences their stability and potential to interact with other clock components such as CRY1 and CRY2 proteins, respectively. Mutations in Cry1 and Cry2 reveal that these two genes have opposite effects on period length. On the basis of this observation and the different temporal dynamics of Per1 and Per2 gene expression, including their differential light responsiveness, the clock mechanism can be seen as an oscillator stabilized by two regulatory loops. One of these regulatory loops reflects a morning oscillator (M) tracking dawn and the other an evening oscillator (E) tracking dusk (Ref. 18, Fig. 1). After interaction, the PER1/CRY1 complex would be transported back into the nucleus through an unknown mechanism, interfere with the CLOCK/BMAL1 complex, and thereby inhibit Per1 and maybe Cry1 transcription. In a similar way, the PER2/CRY2 complex would regulate Per2 and Cry2 transcription.

PER1 can also interact with PER2 directly and influence the amount of PER2 that reaches the nucleus. Therefore a shift in peak expression of Bmal1 should be observed in the Per1 mutant similar to the shift in Per2 mutants. Investigation of Bmal1 expression in Per1 mutants indicates that this is so (6). Hence, PER2 regulates the circadian phase of Bmal1 gene expression and therefore PER1/PER2 interaction seems to be important for regulating the phase relationship between the M and E oscillator (Fig. 1). This will affect other genes that are regulated by the CLOCK/BMAL1 complex as well. Hence, the M and E oscillators are coupled through PER1/PER2 dimerization. A loss of function of one of the Per genes would therefore lead to an impairment in clock synchronization and resetting, which is indeed observed (4). In light-mediated resetting, glutamate plays an important role. Glutamate is released on light stimulation at synapses connecting the eye with the SCN and binds to its receptors on the SCN neuron, thereby activating common signaling pathways. It seems that Per2 light inducibility is mediated by a different signaling pathway than light inducibility for Per1. However, which of the variety of common signaling pathways is activated at a specific circadian time and specifically activate Per1 or Per2 expression is not clear. The specific and differential light responsiveness of the E and M oscillator is illustrated in Fig. 1.

The formal separation of the clock mechanism into an M and an E oscillator is also attractive in view of the finding that the Per1 and Per2 genes are not redundant (82). They can activate clock output genes or ccg separately or in combination. Hence, ccg can be divided into different groups (see above), designated in Fig. 1 as ccg1 (for Per1 dependent), ccg3 (for Per2 dependent), and ccg (for Per1 and Per2 dependent). In addition there could be Per-independent ccg. Whether and how these clock controlled genes feed back to the clock mechanism in SCN neurons is not known.
The model presented in Fig. 1 is showing the presence of the two oscillators in a single SCN neuron. However, it can be argued that the M and E oscillators might be separated and present in different SCN cells. Although this seems not very likely, the fact that SCN neurons are heterogeneous in ultrastructure, cytology, and anatomic connectivity (see Ref. 29a) could favor such a view. Analyses of Clock chimeras have shown that the location of Clock mutant cells influences circadian phenotype (42); therefore, the existence of functional columns in the SCN has been proposed. However, such functional columns could be a consequence of different stimulatory inputs because SCN cells are not equally innervated. This allows the clock to integrate many signals in the whole SCN tissue to create a stable output. For these reasons, it seems unlikely that the proposed M and E oscillators are located in different cells.

Taken together, the model presented here stresses the fact that Per1 and Per2 genes are not redundant and that they can regulate different target genes and opposite behavioral responses. The two regulatory loops driven by Per1 (M) and Per2 (E) are locked into each other, thereby influencing their phase relationship. The Cry genes are the negative regulators in the two loops and are responsible for maintenance of the oscillation. Hence, Per genes would set the phase and Cry genes the period.

OUTLOOK

The human circadian clock is probably regulated in a similar manner as in mice. A short-period circadian rhythm variant in humans has been associated with familial advanced sleep-phase syndrome (33). Families displaying this syndrome have a mutation in a phosphoregulation site of the Per2 gene (73). Other familial syndromes with a disturbed circadian clock that do not cosegregate with Per2 will provide an opportunity to identify mutations in other genes that lead to alteration in human circadian rhythms.

Similar deletions in the Per1 gene have been described in two different mouse strains (6, 82). Although in both mouse strains the Per1 gene is deleted, in one strain a variable period length is observed (82), whereas in the other a loss of circadian rhythmicity is described (6). The difference in the observed behavioral phenotype is probably due to a lack or mutation in a gene in one of the inbred mouse strains that can have a mild effect on the circadian clock. Identification of the difference in these two mouse strains will lead to the discovery of a gene that influences the circadian clock. That additional clock components exist has been suggested by genome-wide epistatic interaction analysis. Complex genetic determinants of circadian behavior in mice have been found. These involve gene loci that are not related to the above-described molecular components of the mammalian clock (67). Therefore, it seems that the investigation of the circadian clock has just seen its dawn, and many genes and even whole biochemical pathways that make up the clock remain to be discovered.

I thank Henrik Oster, Dra. Erik Maronde, Alex Coppell, and Vladan Antic for reading the manuscript. Support from the Swiss National Science Foundation, the German Science Foundation, the Max Planck Society, and the State of Fribourg is gratefully acknowledged.

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