Posttranscriptional Regulation of Mammalian Circadian Clock Output

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Circadian clocks are present in many different cell types/tissues and control many aspects of physiology. This broad control is exerted, at least in part, by the circadian regulation of many genes, resulting in rhythmic expression patterns of 5–10% of the mRNAs in a given tissue. Although transcriptional regulation is certainly involved in this process, it is becoming clear that posttranscriptional mechanisms also have important roles in producing the appropriate rhythmic expression profiles. In this chapter, we review the available data about posttranscriptional regulation of circadian gene expression and highlight the potential role of Nocturnin (Noc) in such processes. NOC is a deadenylase—a ribonuclease that specifically removes poly(A) tails from mRNAs—that is expressed widely in the mouse with high-amplitude rhythmicity. Deadenylation affects the stability and translational properties of mRNAs. Mice lacking the Noc gene have metabolic defects including a resistance to diet-induced obesity, decreased fat storage, changes in lipid-related gene expression profiles in the liver, and altered glucose and insulin sensitivities. These findings suggest that NOC has a pivotal role downstream from the circadian clockwork in the posttranscriptional regulation genes involved in the circadian control of metabolism.

INTRODUCTION

Daily oscillations in mRNA levels are a distinctive feature of circadian rhythms. Approximately 1–10% of the transcripts expressed in a particular cell/tissue are under circadian control, as observed in mammals, birds, insects, plants, fungi, and cell cultures (Duffield 2003; Sato et al. 2003; Lowrey and Takahashi 2004). In cyanobacteria, expression of the entire genome appears to be modulated by circadian clocks (Liu et al. 1995; Woelfle and Johnson 2006). The regulation of mRNA levels constitutes an intrinsic component of the molecular clock function and seems to be a widespread mechanism by which these oscillators control key enzymes, transcription factors, and regulators governing biosynthetic and metabolic pathways. As a consequence of these oscillations, several systemic signals, as well as many of the receptors required to decode these signals, are periodically produced for the orchestration of physiological and behavioral rhythms.

It is obvious that the abundance of a specific mRNA or protein depends on the balance between its synthesis and degradation. However, with regard to circadian oscillations of mRNA levels, there are two major prejudices that instinctively arise: Rhythmic transcription controls mRNA oscillations, and messenger content reflects the level and activity of the corresponding protein. These suppositions are true in many cases, but not always, and in general, other mechanisms contribute significantly to regulate both mRNA and protein levels. The levels of a particular mRNA can decrease even when its transcription increases if degradation rates increase more, and the abundance of a protein can change when its transcript level remains constant if its translation is silenced or enhanced. These apparent paradoxes are not just rhetorical speculations; there are many such examples that have been documented (see, e.g., Hastings 2001; Shu and Hong-Hui 2004; Reddy et al. 2006).

The control of gene expression is a complex process comprising several steps that are tightly regulated: (1) transcription, (2) mRNA processing (capping, splicing, polyadenylation, and quality control), (3) nuclear export, (4) sorting and transport (whereas most mRNAs are immediately translated, others are stored or translocated to specific cellular regions), (5) translation, and (6) mRNA degradation (Fig 1). Presently, we have evidence showing that some of these steps are regulated by clocks, but in theory, all of these events are possible points of circadian modulation.

Despite the considerations discussed above, it is important to point out that transcription is thought to be the primary source of many transcript oscillations (Harms et al. 2004). However, one should be careful before assuming it for a specific mRNA, and in general, transcriptional and posttranscriptional processes act coordinately in the regulation of expression programs. During recent years, it has become increasingly clear that mRNA decay and translational regulation have important consequences on gene expression and are tightly regulated (Wilusz et al. 2001; Gebauer and Hentze 2004; Wilusz and Wilusz 2004; Eulalio et al. 2007; Garneau et al. 2007; Mathews et al. 2007; Parker and Sheth 2007; Pillai et al. 2007). Microarray studies have shown that regulation of mRNA stability may account for as much as 50% of the variations in poly(A) mRNA levels and has a major role in regulating expression programs (for review, see Raghavan and Bohjalian 2004; Cheadle et al. 2005; Mata et al. 2005). Transcription has been extensively studied from a chronobiologic perspective, whereas posttranscriptional mechanisms have received much less attention.

Recently, we demonstrated that the rhythmic gene Nocturnin (Noc) encoded a deadenylase, an exoribonuclease specific for poly(A) tails of mRNAs (Green and Besharse 1996a; Baggs and Green 2003). To date, this is the only ribonuclease known to be under circadian control,
providing a possible mechanism for rhythmic posttranscriptional control. In this chapter, we review what is currently known about the role of posttranscriptional regulation in shaping circadian gene expression patterns and discuss potential roles of Noc in these events. Posttranslational regulatory mechanisms are not discussed here.

EXEMPLARY OF CIRCADIAN POSTTRANSCRIPTIONAL REGULATION

As stated above, posttranscriptional regulation includes numerous events (Fig. 1), many of which have not been approached from a chronobiologic perspective at all, and in general, we have a fragmented vision of how clocks may act at this level. Clock modulation of mRNA nuclear processing (including capping, splicing, polyadenylation, and messenger quality control), nuclear export, and sorting for storage or specific localization has not been examined. However, there are a number of reports that link posttranscriptional events to clock function. In one interesting example, the loss of function of Fragile-X mental retardation protein (FMRP), an RNA-binding protein (RBP), produces defects in clock output in Drosophila (Dockendorff et al. 2002). In addition, several examples demonstrate a role for regulation of mRNA decay and translation in circadian gene expression (summarized in Table 1). We review some of these examples in this section.

**Arginine Vasopressin mRNA Poly(A) Length**

Two decades ago, a daily regulation of vasopressin (Avp) mRNA poly(A) tail length in rat suprachiasmatic nuclei (SCN), but not in other brain areas, was reported (Robinson et al. 1988). The transcript levels of Avp are circadianly regulated specifically in SCN, showing the lowest levels at night (Uhl and Reppert 1986; Cagampang et al. 1994), when the mRNA species with short poly(A) tail length is observed (Robinson et al. 1988). Nuclear run-on experiments showed an approximately 30% diminution in transcription rate of Avp at night (Carter and Murphy 1992), which did not account for the total changes in mRNA levels. Because deadenylation (shortening of the poly(A) tail) was known to often trigger mRNA decay, these observations suggest that Avp mRNA decay rates are higher when the poly(A) tail is shorter, contributing to the rhythm of steady-state Avp transcript levels. On the other hand, Avp mRNA is translocated to dendrites for local synthesis (Mohr and Richter 2004), so the changes in poly(A) length may also influence this phenomenon, affecting the temporal production of this neuromodulator in synaptic regions.
### Table 1. Factors Involved in Posttranscriptional Regulation of Circadian Expression

<table>
<thead>
<tr>
<th>Factor</th>
<th>Organism</th>
<th>Motif (CDD#)</th>
<th>Function</th>
<th>References</th>
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<tr>
<td>Enzymes</td>
<td></td>
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<tr>
<td>NOCTURNIN (CCRN4L)</td>
<td><em>Xenopus laevis</em>; <em>Mus musculus</em></td>
<td>endonuclease/ exonuclease/ phosphatase (pfam03372)</td>
<td>deadenylase (poly(A)-specific ribonuclease); likely regulates the mRNA decay and/or translational properties of circadian-related transcripts; mNoc&lt;sup&gt;−&lt;sup&gt;/&lt;/sup&gt;−&lt;/sup&gt; mouse has metabolic alterations</td>
<td>Baggs and Green (2003); Garbarino-Pico et al. (2007); Green et al. (2007)</td>
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<tr>
<td>RNA-binding proteins</td>
<td></td>
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<tr>
<td>AtGRP7</td>
<td><em>Arabidopsis thaliana</em></td>
<td>RNA recognition motif (RRM) (smart00360)</td>
<td>regulator of Atgrp7 and Atgrp8 mRNAs splicing; produces an unstable variant at specific times; involved in the regulation of abscisic acid and stress responses</td>
<td>Heintzen et al. (1997); Staiger et al. (2003); Cao et al. (2006)</td>
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<tr>
<td>CCTR</td>
<td><em>Lingulodinium polyedrum</em> (formerly Gonyaulax)</td>
<td>n.d.</td>
<td>binds rhythmically to the 3´UTR (UG-repeat region) of luciferin-binding protein mRNA, repressing its translation and indirectly regulating bioluminescence rhythms</td>
<td>Mittag et al. (1994); Mittag (2003)</td>
</tr>
<tr>
<td>CHLAMY1</td>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>3x K homology RNA-binding domain; type I (KH-I) (cd00105); two conserved tryptophan domain (WW) (cd00201); and 3x RRM (smart00360)</td>
<td>binds rhythmically to 3´UTRs (UG-repeat region) of genes involved in N&lt;sub&gt;2&lt;/sub&gt; and CO&lt;sub&gt;2&lt;/sub&gt; metabolism; also implicated in the control of the phase angle and period of the circadian clock</td>
<td>Mittag (1996); Waltenberger et al. (2001); Zhao et al. (2004); Iliev et al. (2006)</td>
</tr>
<tr>
<td>hnRNP L</td>
<td><em>Rattus norvegicus</em></td>
<td>3x RRM (smart00360)</td>
<td>induces nocturnal Aanat mRNA degradation by binding cis-acting elements in its 3´UTR</td>
<td>Kim et al. (2005)</td>
</tr>
<tr>
<td>hnRNP Q</td>
<td><em>Rattus norvegicus</em></td>
<td>3x RRM (smart00360)</td>
<td>similar to hnRNP Q, but in addition binds another site in 5´UTR increasing Aanat translation rate</td>
<td>Kim et al. (2005, 2007)</td>
</tr>
<tr>
<td>hnRNP R</td>
<td><em>Drosophila melanogaster</em></td>
<td>3x RRM (smart00360); 2x RRM (smart00360); CCHC-type zinc finger (smart00343)</td>
<td>shows constant mRNA levels, but rhythmic protein accumulation; affects daily timing of adult eclosion but not clock properties or input mechanism</td>
<td>Kim et al. (2005); Newby and Jackson (1993, 1996); McNeil et al. (1998); Schroeder et al. (2003); Kojima et al. (2007)</td>
</tr>
<tr>
<td>LARK</td>
<td><em>Mus musculus</em></td>
<td>2x RRM (smart00360); CCHC-type zinc finger (pfam00098)</td>
<td>also shows constant mRNA levels, but rhythmic protein accumulation; binds 3´UTR of mPer1 transcript enhancing its translation; its up- or down-regulation affects period in cell cultures</td>
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<tr>
<td>Regulatory RNAs</td>
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<tr>
<td>Antisense Frq mRNAs</td>
<td><em>Neurospora crassa</em></td>
<td>n.a.</td>
<td>cycles in antiphase with sense Frq mRNA and is induced by light; affects circadian rhythms</td>
<td>Kramer et al. (2003)</td>
</tr>
<tr>
<td>miR-219</td>
<td><em>Mus musculus</em></td>
<td>n.a.</td>
<td>expression is circadianly regulated by CLOCK/BMAL1; its knockdown lengthens period it is induced by light, attenuating its entraining effects</td>
<td>Cheng et al. (2007)</td>
</tr>
<tr>
<td>miR-132</td>
<td><em>Mus musculus</em></td>
<td>n.a.</td>
<td></td>
<td>Cheng et al. (2007)</td>
</tr>
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<sup>a</sup>Conserved domain database, NCBI (Marchler-Bauer et al. 2007). n.d. indicates not determined; n.a. indicates not applicable.

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**Circadian Regulation of Splicing Variants**

The *Arabidopsis* AtGRP7 is a circadian-expressed RBP that regulates the alternative splicing of its own message and, as consequence, its own expression rhythm (Staiger et al. 2003). When the protein accumulates, AtGRP7 binds a site in the 3´UTR (untranslated region) of its pre-mRNA that would induce a shift in splice site selection; the variant produced contains a premature stop codon and is very unstable, causing the reduction of the Atgrp7 mRNA and protein levels. Additionally, AtGRP7 has a similar effect on the splicing of the Atgrp8 transcript and may act on other gene products as well (Staiger et al. 2003).

The murine Presenilin2 gene expresses different mRNA splice variants; two of them are rhythmic, whereas a third one is not (Belanger et al. 2006). It is not clear whether this is caused by circadian modulation of spliceosome machinery or of the splice variant stability. *Drosophila Period* (*Per*) represents another interesting example. It exhibits daily regulation in the splicing of an intron in the 3´UTR that shows seasonal changes...
Period (Per) mRNA

Frish et al. (1994) found that the promoter of Drosohila Per is not required for its circadian expression. This demonstration of a transcription-independent mRNA rhythm was particularly surprising because Per is part of the molecular clock machinery. The delivery of a promoterless-dPer transgene can actually restore locomotor activity oscillations in arrhythmic flies (Frish et al. 1994). However, the amplitude of the mRNA content rhythm in the transgenic flies is significantly lower, suggesting that transcription does contribute to the normal oscillation. Indeed, the transcription rate of dPer shows a high-amplitude oscillation (Hardin et al. 1992; So and Rosbash 1997). In an elegant study, So and Rosbash (1997) showed that both transcriptional and posttranscriptional events regulate dPer transcript rhythms. These authors compared the transcription rate temporal profile measured by nuclear run-on assays, with the mRNA content oscillation determined by RNase protection assays. This comparison revealed that the half-life of the dPer mRNA changes between twofold and fourfold over the course of the circadian cycle, demonstrating that the dPer mRNA decay rate is under circadian control. (The same study also showed that posttranscriptional events are involved in the generation of the mRNA rhythm of a clock-controlled gene called Crg-1.) dPer mRNA contains elements in its 5′ and 3′UTRs that affect PER protein cycling and fly behavior, probably through regulation of mRNA stability (Chen et al. 2002). Additionally, it has been shown that translation is also under circadian regulation; two alternative splicing variants exhibit different translational properties, and cyclic dPer mRNA is not required for PER protein rhythmicity (Cheng et al. 1998; Cheng and Hardin 1998).

The mammalian homologs of dPer also have been shown to be regulated at the posttranscriptional level. The 3′UTR of human and murine Per1 transcripts exhibit a high degree of homology, and in the mouse, this region has been shown to be involved in the regulation of mPer1 expression (Wilsbacher et al. 2002; Kojima et al. 2003). mLARK, a circadian expressed RBP, binds an element in the mPer1 3′UTR resulting in enhanced translation (Kojima et al. 2007). Remarkably, both dLARK and mLark show constant mRNA levels but rhythmic protein accumulation, denoting circadian posttranscriptional regulation of their expression (Newby and Jackson 1996; McNeil et al. 1998; Kojima et al. 2007).

The other two mammalian Per genes also have been shown to be regulated at the posttranscriptional level. mPer2 expressed constitutively in NIH-3T3 cultures exhibits cyclic protein levels even while showing constant mRNA content, likely due to translational and/or proteolytic regulation (Yamamoto et al. 2005; Fujimoto et al. 2006; Nishii et al. 2006). The 3′UTR of mPer3 mRNA also possesses a regulatory element(s) controlling its stability, but the trans-acting factor(s) responsible for this phenomenon has not yet been identified (Kwak et al. 2006).

Aryalkylamine N-acetyltransferase mRNA

The regulation of Aanat (aryalkylamine N-acetyltransferase) expression, a key enzyme in melatonin biosynthesis, constitutes another example of circadian control at multiple levels, including transcription (Baier et al. 1997; Foulkes et al. 1997), mRNA destabilization (Kim et al. 2005), and translation (Kim et al. 2007). Kim et al. (2005) found that the Aanat mRNA 3′UTR possesses destabilization elements. The circadianly expressed heterogeneous nuclear ribonucleoprotein (hnRNP) R, hnRNP Q, and hnRNP L induce the nocturnal Aanat mRNA degradation by binding those cis-acting elements (Kim et al. 2005). Furthermore, the 5′UTR of rat and sheep Aanat mRNAs contain an internal ribosome entry site (IRES) that confers rhythmic translation (Kim et al. 2007). IRESs allow ribosomes to bind transcripts and initiate translation in an alternative mechanism to the canonical cap-dependent scanning model (Jackson 2005; Elroy-Stein and Merrick 2007). hnRNP Q was also identified as one of the trans-acting factors responsible for the circadian activation of Aanat translation (Kim et al. 2007). Its expression levels, as well as its binding to the Aanat mRNA 5′UTR, oscillates concomitantly with AANAT protein abundance.

Other Examples Involving mRNA Decay in Circadian Control of Gene Expression

In addition to the previous examples, several other mRNAs have been shown to be regulated posttranscriptionally; however, the mechanisms and trans-acting factors involved have not been identified. Studies done in Arabidopsis thaliana have provided several examples of mRNA rhythms controlled at transcript stability level: Chlorophyll a/b binding 1 (Millar and Kay 1991), Nitrato reductase 2 (Pilgrim et al. 1993) and Ccr-like, and SeneScience associated gene 1 (Lidder et al. 2005). Interestingly, some Arabidopsis clock-controlled genes (ccgs) which have mRNAs that are unstable contain a downstream (DST) element (apparently unique to plants) in their 3′UTR that confers the rapid decay property, but the factor acting on this element has not been characterized (Gutierrez et al. 2002; Lidder et al. 2005). In maize, two Rubisco activase genes expressing mRNAs with the same coding region but different 3′UTRs have been reported (Ayala-Ochoa et al. 2004). Both show daily changes in their levels but different degradation rates, presumably because distinct DST-like elements are present in their 3′UTRs. Rice CatalaseA (CatA) expression also was suggested to be regulated at the posttranscriptional level (likely via pre-mRNA stability), because a transgenic reporter gene driven by the promoter of CatA, showed different temporal patterns of mRNA levels when compared with the endogenous transcript (Iwamoto et al. 2000). However, it could not be ruled out that other gene regions outside the promoter affect transcription.
Circadian-regulated RBPs in Microalgae

In the dinoflagellate Lingulodinium polyedrum (formerly Gonyaulax) and the green algae Chlamydomonas reinhardtii, two related circadian-controlled RBPs were identified (for review, see Mittag 2003). In L. polyedrum, the circadian-controlled translational regulator (Cctr) protein product binds rhythmically to the 3’UTR of the luciferin-binding protein (lbp) mRNA regulating its translation and, consequently, the bioluminescence rhythm (Mittag et al. 1994). Using the lbp 3’UTR as bait, CHLAMY 1 was later identified in C. reinhardtii (Mittag 1996). Although this microalga does not express lbp, other mRNA targets were identified that contain a cis-acting element related to the one in lbp. The CHLAMY 1 targets include enzymes and factors involved in nitrogen and carbon dioxide metabolism that are circadianly regulated (Waltenberger et al. 2001). CHLAMY 1 was also implicated in the control of the phase angle and period of the circadian clock (Iliev et al. 2006).

Circadian Regulation of Translation

In general, the examples of this kind of regulation are inferences from comparisons between mRNA and protein profiles, thus little mechanistic information is known. However, some RBPs involved in translational control have been identified (see above LARK, hnRNP Q, CCTR, and CHLAMY 1). Woodland Hastings’s laboratory has been the pioneer in studying circadian posttranscriptional regulation in the dinoflagellate L. polyedrum (for review, see Hastings 2001). His laboratory showed that the accumulation of several proteins exhibit oscillations generated at the translational level and also was the first to identify a circadian-regulated RBP (CCTR, see above). In this organism, total protein synthesis in both cytoplasm and chloroplast shows circadian changes (Donner et al. 1985). Markovic et al. (1996) showed tenfold circadian changes in the synthesis rates of several proteins that exhibit constant levels, suggesting that this regulation is required for temporal organization of translation, rather than for generating a protein oscillation.

The Neurospora clock protein FRQ exists in two forms as a result of the use of alternative translation initiation sites (Garneau et al. 1997). And in Arabidopsis, the levels of the transcription factor LHY increase concurrently with the down-regulation of its mRNA during light induction, presumably acting to narrow the peak of this protein involved in the molecular clock (Kim et al. 2003).

Rhythmic Posttranscriptional Control by Noncoding RNAs

Recently, noncoding regulatory RNAs have been implicated in several functions related to mRNA metabolism, but there are only a few studies thus far involving them in circadian rhythms. In Neurospora, sense and antisense Frq mRNAs were identified that cycle in antiphase, and all are induced by light. Mutant strains not expressing these antisense transcripts show altered rhythms (Kramer et al. 2003). Natural antisense RNAs have also been found for mammalian Rev-erbα and Antheraea pernyi (silkmoth) Per (for discussion, see Crosthwaite 2004). A recent study reported for the first time the circadian regulation of a microRNA (miRNA); miR-219 rhythm is driven by CLOCK/BMAL1 and its knockdown lengthens circadian period (Cheng et al. 2007). Another miRNA, miR-132, responds to light affecting entrainment (Cheng et al. 2007).

Nocturnin

Finally, the recent determination that the rhythmic Noc gene encodes a deadenylase provides another mechanism for regulating circadian posttranscriptional output (Baggs and Green 2003). This is discussed in more detail below following a summary of the major mechanisms involved in mRNA turnover.

mRNA Decay Pathways

Many recent advances have been made in the understanding of mRNA-decay mechanisms and several excellent reviews on this topic have recently been published (Witusz et al. 2001; Parker and Song 2004; Witusz and Witusz 2004; Garneau et al. 2007). In particular, the involvement of noncoding regulatory RNAs and the founding of novel cytoplasmic subdomains are revolutionizing the field and rapidly changing our view of mRNA degradation and translation control (Anderson and Kedersha 2006; Valencia-Sanchez et al. 2006; Eulalio et al. 2007; Mathews et al. 2007; Parker and Sheth 2007; Pillai et al. 2007).

Although a nascent transcript is still being synthesized, it is coated with regulatory factors including RBPs, enzymes, and noncoding regulatory RNAs. These complexes—ribonucleoparticles or ribonucleoproteins (mRNPs)—are very dynamic and change their composition with respect to the different functions associated with pre-mRNA processing, transcript translocation, translation, in some cases storage or specific localization, and finally, degradation (Fig. 1). Importantly, transcripts contain regulatory elements along their body including the 5’-methylguanosine cap (m7Gppp), the 5’UTR, the coding sequence, the 3’UTR, and the poly(A) tail. Generically, they are called cis-acting elements and constitute the sites for interacting with RBPs, the trans-acting factors. A picture of mRNA posttranscriptional processing is emerging that is analogous to the combinatorial model of transcription regulation: Whereas the activation of a gene depends on the arrangement of transcription factors bound to regulatory sites in its promoter, the fate of an mRNA depends on the combination of RBPs and noncoding regulatory RNAs associated with their cis-acting elements (Keene 2007). These factors recruit the enzymes and other regulatory and scaffold proteins that process the mRNA and control its fate.

The half-life of different mRNA species varies between minutes and days, some of them survive for only fractions of the cell cycle, whereas others last for many divisions. The stability of a particular transcript can change in different tissues or physiological conditions, in response to stimulation, across development, or along circadian time, as was exemplified in the previous section. In general, the levels of an mRNA correlate well with its translation.
rates; however, the number of examples where messages leave polysomes and are stored without being degraded is increasing. This constitutes a fast and economic mechanism for silencing the expression of a gene and such a message can later resume its translation independently of transcription and nuclear processing. For example, during the stress response, the translation machinery is co-opted to synthesize the factors involved in the cellular response, and the majority of the non-stress-related transcripts are stored in stress granules until their translation is again necessary (Anderson and Kedersha 2002).

In addition to participating in regulation of expression levels, mRNA decay is also involved in the antiviral defense and quality control of transcripts. These processes include double-stranded RNA responses or RNA interference, non-sense-mediated decay (NMD), nonstop decay (NSD), and nogo decay (NGD); these are not discussed here. The pathways responsible for “normal” mRNA decay comprise deadenylation-dependent mRNA decay, deadenylation-independent mRNA decay, and endonuclease-mediated mRNA decay.

The cap and the poly(A) structures stabilize transcripts by their interaction with translation initiation factor 4E (eIF4E) and poly(A)-binding protein (PABP), respectively, protecting against exonuclease access and enhancing translation. The poly(A) tails of eukaryotic mRNAs usually contain 25–200 residues that interact with a number of regulators in addition to PABP (Mangus et al. 2003; Kuhn and Wahle 2004). It is currently believed that deadenylation-dependent mRNA decay is the pathway by which most transcripts are degraded in eukaryotic cells (Cao and Parker 2001). It initiates with the enzymatic shortening of the poly(A) tail, which is performed by deadenylases. The deadenylation step is very important because it is believed to constitute the rate-limiting step and the more-regulated step in the turnover of most transcripts. In addition, it is implicated in translational silencing. Several deadenylases have been identified (for review, see Parker and Song 2004); however, not much is known about deadenylase regulation and specificity. Some of these enzymes’ activities are modulated by the presence or absence of the 5′ cap and/or the presence or absence of PABP in in vitro reactions. The spatial and temporal distribution of each deadenylase is another obvious aspect to consider and may impact the in vivo substrate specificity and the function of these enzymes. Recently, some RBPs and miRNAs have been implicated in the recruitment of deadenylases to specific targets.

After deadenylation, mRNA can be degraded in 3′→5′ direction by the exosome, a complex containing exonucleases and accessory proteins (Liu et al. 2006), followed by hydrolysis of the remaining m7Gppp cap by the scavenger-decapping enzyme DCP2. Alternatively, the deadenylated messenger can be degraded in 5′→3′ direction being first decapped by DCP1 and DCP2 and then digested by the exoribonuclease XRN1.

Two other degradation pathways have been reported but appear to be much less common. Deadenylation-independent mRNA decay is triggered by decapping by DCP1 and DCP2, followed by degradation by XRN1. Endonucleolytic degradation bypasses the two major transcript stabilizers (cap and tail) by generating endonucleolytic products lacking the 5′ m7Gppp or the 3′ poly(A), which are therefore susceptible to degradation by XRN1 and the exosome, respectively. The few ribonucleases described thus far are associated with specific cellular compartments (i.e. endoplasmic reticulum, mitochondria, or polysomes) and are tightly regulated.

The discovery of new cytoplasmic subdomains where mRNPs accumulate, and a better understanding of the composition and function of previously described RNA granules, has modified our view of cytoplasmic mRNA metabolism and has provided a place for physical interaction between translation and mRNA decay factors (for review, see Anderson and Kedersha 2006; Kiebler and Bassell 2006; Eulalio et al. 2007; Parker and Sheth 2007). These RNA granules include processing bodies (PBs), also known as GW or DCP1 bodies, stress granules (SGs), neuronal granules, and maternal or germinal granules. They contain translationally silenced mRNAs, RBPs, translation and RNA-degradation factors, scaffold proteins, and, in some cases, ribosomal subunits, miRNAs, and components of miRNA machinery. SGs and PBs are very dynamic structures that respond to physiological stimulation. It has been shown that transcripts can be redirected from polysomes to SGs and PBs or vice versa in response to different conditions, and as a consequence, the message is translated, stored, or degraded. PBs have been reported in yeast and mammalian cell cultures, but it is still not clear whether they have a prominent function in vivo. Importantly, in addition to their presence in PBs and SGs, the components of these structures are also dispersed in cytoplasm, and assembly of these foci is not a requisite for translational silencing, mRNA turnover, or storage.

There is considerable evidence for an interrelationship between translation and mRNA decay (for review, see Schwartz and Parker 2000). In general, when the translation of an mRNA is optimized, its turnover is decreased, and conversely, factors that induce transcript decay may diminish translation efficiency. Models have been proposed where the translation factors associated with the poly(A) tail and cap protect the transcript from deadenylases and decapping enzymes through competition or inhibition (Schwartz and Parker 2000). Another related observation is that PBs and SGs are disrupted by polysome stabilizers (i.e., cycloheximide) and their number and size increased by drugs that disassemble polysomes (i.e., puromycin). However, little is known about the mechanisms dictating when an mRNA switches from interacting with translation factors and ribosomes to being trapped by the decay machinery.

Discovery of Nocturnin

Noc was identified in a differential display screen performed in Xenopus retina searching for circadianly controlled mRNAs (Green and Besharse 1996a,b). It was named nocturnin by virtue of the high nocturnal increase that its transcript shows in the frog eye (Green and Besharse 1996a). The cDNA encoded a novel protein of 388 amino acids. Our laboratory initially studied this gene in Xenopus in an attempt to establish its function and significance in cir-
CIRCADIAN POSTTRANSCRIPTIONAL CONTROL

MOUSE NOCTURNIN IS CIRCADIANLY REGULATED AND BROADLY EXPRESSED

Noc is a Mg²⁺-dependent poly(A)-specific ribonuclease (deadenylase). Baggs and Green (2003) demonstrated that, like yCCR4, it also exhibits deadenylase activity (Green and Besharse 1996a). The nocturnal increase of xNoc transcription is caused by the binding of phosphorylated CREB to a noncanonical CRE identified in the xNoc promoter (Liu and Green 2001). The xNoc protein accumulation also is circadian-regulated in Xenopus retinas, consistently with the mRNA oscillation described (Baggs and Green 2003).

Originally, the only information we had regarding xNoc function was the spatial and temporal expression pattern described above, and its sequence. The carboxyl terminus of xNoc exhibits high similarity with the same region of the protein product of yeast Carbon catabolite repression 4 (yCcr4) (Green and Besharse 1996a). For this reason, Noc is also known as Carbon catabolite repression 4-like (Ccrn4). yCcr4 is a subunit of the CCR4-NOT complex, a transcription factor involved in carbon metabolism regulation. To analyze whether xNoc was also a transcription factor, we determined the subcellular localization of both native and overexpressed tagged proteins. They were never found in the nucleus; xNoc was systematically located in cytoplasm (Baggs and Green 2003). Later, it was shown that yCcr4 also exhibits deadenylase activity (Chen et al. 2002; Tucker et al. 2002). After testing alternative hypotheses regarding xNoc biochemical function, Baggs and Green (2003) demonstrated that, like yCcr4, it is a Mg²⁺-dependent poly(A)-specific ribonuclease (deadenylase). Noc belongs to the endonuclease/exonuclease/phosphatase family that includes DNase I, APE1, IP5P, CCR4, and CCR4-related proteins (Dlakic 2000; Dupressoir et al. 2001). Considering the xNoc temporal expression profile, the finding that xNoc is a deadenylase led to the formulation of the hypothesis that it regulates circadian gene expression at a posttranscriptional level by inducing the degradation or translational silencing of clock-related mRNAs (Baggs and Green 1999; Wang et al. 2001). The message also possesses a conserved second ATG site that meets Kozak consensus requirements which might act as an alternative translation initiation site resulting in a protein of 365 amino acids and 41 kD (Wang et al. 2001). Western blots performed with two alternative antibodies generated against mNoc showed a unique band in mouse fibroblast (Garbarino-Pico et al. 2007) and tissue (E. Garbarino-Pico and C.B. Green, unpubl.) lysates. The electrophoretic mobility of the protein detected favors the possibility that the actual translation initiation site is the second one; however, this evidence is not conclusive and further experiments are required in order to resolve this issue.

The carboxy-terminal region of mNoc has a high degree of identity with the catalytic domain of yCcr4. In contrast, the amino-terminal regions of these proteins are dissimilar; mNoc lacks the two transcriptional activation domains and the leucine-rich repeat region of CCR4 (Dupressoir et al. 2001; Wang et al. 2001). The absence of these transcriptional activation domains and its cytoplasmic localization (J.E. Baggs et al., unpubl.) rule out the possibility that mNoc has a role as a transcription factor. As expected, mNoc (like xNOC) exhibits deadenylase activity (Garbarino-Pico et al. 2007). Molecular exclusion chromatography and immunoprecipitation studies have shown that mNoc is included in complexes with other proteins (J.E. Baggs et al., unpubl.); it is likely that these partners have a role in modulating and targeting mNOC activity in vivo.

As in Xenopus, mNoc expression is also under circadian control showing high-amplitude rhythms in mRNA levels (Wang et al. 2001; Barbot et al. 2002) and protein (E. Garbarino-Pico and C.B. Green, unpubl.) levels. The wide spatial distribution of Noc in mouse contrasts with its localized expression in frog. The mNoc transcript has been detected in most mouse tissues, including brain, colon, heart, intestine, liver, lung, kidney, ovary, spleen, testis, thymus, and retina (Dupressoir et al. 1999; Wang et al. 2001; Barbot et al. 2002), as well as in fibroblast cultures (Garbarino-Pico et al. 2007). In the central nervous system, Noc mRNA was observed in the hypothalamus, SCN and arcuate nucleus, olfactory bulb, piriform cortex, hippocampus, subiculum, cerebellum, and pineal gland (Wang et al. 2001). The broad expression in the mouse suggests that mNoc has a role in regulating transcripts involved in a wide range of functions.

mNoc IS AN IMMEDIATE-EARLY GENE

While investigating mNoc expression in NIH-3T3 cell cultures, we found that it is acutely induced by extracellular stimuli. We tested the effect of a serum shock, the phorbol ester TPA, forskolin, and dexamethasone because of their capabilities of entraining circadian clocks in fibroblast cultures (for review, see Nagoshi et al. 2005). mNoc mRNA levels show an approximately 30-fold increase after 2 hours of serum shock compared with serum-starved quiescent NIH-3T3 cells. This induction is also reflected in the protein levels which show an approximately ninefold induction 2.5 hours after the stimulation. Remarkably, the other four deadenylases reported in the
mouse are not induced by this treatment. TPA also acutely stimulates \( m_{\text{Noc}} \) transcript accumulation, but forskolin and dexamethasone do not (Garbarino-Pico et al. 2007).

The TPA \( m_{\text{Noc}} \) acute induction is independent of protein synthesis and transient, with both the transcript and protein having a short half-life (Garbarino-Pico et al. 2007). These properties define an immediate-early gene (IEG): These genes primarily and directly respond to a stimulus or physiological change without requiring the synthesis of transcription factors or other regulators. Indeed, IEGs are the factors responsible for driving the cell response to new conditions through regulation of the expression of other genes, suggesting that \( m_{\text{Noc}} \) is involved in such an adaptive response to stimuli.

Because the change of expression programs involves the turning on of some genes and the silencing of others, it is natural that in addition to transcription factors, mRNA decay and silencing factors should also be involved in these responses. But again, as for circadian gene expression, the participation of transcription in response to extracellular stimulation has been extensively studied, whereas mRNA decay and translation considerably less so.

The acute response of \( m_{\text{Noc}} \) to extracellular signals raises the question of whether its rhythmic expression is generated autonomously by the intracellular molecular clock or by a circadian systemic signal (note that these alternatives are not mutually exclusive). We still do not have a conclusive answer to this question, but several observations suggest that a daily induction by an extracellular factor, rather than by the intracellular clockwork, regulates \( m_{\text{Noc}} \) circadian expression: (1) \( m_{\text{Noc}} \) mRNA peaks at the same time in different tissues (Wang et al. 2001), whereas clock genes have different phases in different organs. (2) \( m_{\text{Noc}} \) and \( Dbp \) (a \( \text{CLOCK/BMAL1} \)-acting gene) transcripts have different phase relationships in liver when comparing animals kept under a light/dark cycle versus constant darkness (Barbot et al. 2002). (3) Whereas two agents that entrain circadian clocks in cell cultures induce \( m_{\text{Noc}} \) (serum and TPA), two others do not (forskolin and dexamethasone); in addition, the TPA induction does not require the synthesis of clock proteins (Garbarino-Pico et al. 2007). (4) Mice deprived of food for 1 day and maintained in constant light show a significantly lower nocturnal \( m_{\text{Noc}} \) mRNA increase compared with controls (Barbot et al. 2002). (5) In transgenic mice in which the liver molecular clock function was specifically impaired, most liver clock-controlled genes lost their rhythmicity; however, \( m_{\text{Noc}} \) is among the few mRNAs that still oscillate, presumably being driven by rhythmic systemic signals (Kormann et al. 2007).

Many humoral factors, metabolites, and physiological signals show circadian oscillations in metazoans as a consequence of the feeding, activity, drinking, hormonal, and temperature rhythms (for discussion, see Schibler et al. 2003). It is not clear whether any of them induce \( m_{\text{Noc}} \) expression. Interestingly, lowering blood pH in mice causes an acute induction of \( Noc \) expression in kidney proximal tubules (P.A. Preisig, pers. comm.). Both serum and TPA trigger many signaling cascades; thus, it is not possible to make a strong prediction about what induces \( m_{\text{Noc}} \) in vivo. Sequence analysis of the promoter region of \( m_{\text{Noc}} \) shows putative binding sites for SP1 and NF-κB (Dupressoir et al. 1999; M. Hurt and C.B. Green, unpubl.). There are also E-box sequences and other potential elements in the \( m_{\text{Noc}} \) promoter region, but none of them have been functionally tested.

### \( m_{\text{Noc}} \) AND METABOLISM

To understand the role of \( Noc \) in vivo, mice lacking \( Noc \) (\( Noc \) KO) were generated by homologous recombination (Green et al. 2007). Homozygous \( Noc \) KO mice have normal circadian locomotor rhythms and normal expression of the core clock genes, suggesting that \( Noc \) is not part of the core circadian mechanism in mice. However, these mice have a striking metabolic phenotype, characterized by resistance to diet-induced obesity and resistance to hepatic steatosis. In addition, these animals have alterations in glucose and insulin sensitivity and in thermogenesis. As mentioned above, \( Noc \) is normally expressed with extremely high-amplitude rhythms in the liver, and examination of gene expression showed a number of changes in the profiles of key genes involved in lipid synthesis and/or utilization, several of which are themselves rhythmic. Many aspects of metabolism are known to be under the control of circadian clocks, and NOC presumably contributes to the clock’s control of these processes through its impact on the posttranscriptional regulation of cycling mRNAs. Interestingly, a recent study by Reddy et al. (2006) found that almost the 50% of the rhythmic proteins in liver are translated from mRNAs with constitutive steady-state levels. This surprising result suggests that extensive circadian posttranscriptional control may be occurring in liver. The effect of \( Noc \) on liver metabolism may be through contributions to these types of processes.

### CONCLUSIONS

There is an increasing body of evidence involving posttranscriptional control in the regulation of circadian clock output. However, we are still far away from knowing to what extent posttranscriptional events can contribute to circadian gene expression. This is due in part because we usually measure steady-state level of mRNAs and proteins rather than transcription, mRNA decay, or translation rates. It has been shown that about 2–6% of \( \text{Arabidopsis} \) transcripts are under circadian control by using microarray technology (Harmer et al. 2000; Schaffer et al. 2001), surprisingly, when changes in transcriptional rates were measured by promoter-trap experiments, about 35% of the promoters were shown to be rhythmically controlled (Michael and McClung 2003). As described above, several circadian transcripts are already known to contain \( \text{cis} \)-acting elements interacting with circadian regulated RBPs, providing a mechanism to understand how clocks can control mRNA degradation or translation. Indeed, it may be that there is a specific \( \text{cis} \)-acting element(s) and a RBP(s) responsible for circadian mRNA degradation or translation, in a manner analogous to how \( \text{CLOCK/BMAL1} \) acts on E boxes in the promoters of clock-controlled genes.
The identification of NOC as a deadenylase provides one mechanism by which the circadian clock can posttranscriptionally regulate gene expression. NOC’s expression patterns are unique among the known deadenylases, suggesting that it acts on specific target mRNAs that are important to the circadian system or in response to acute stimulation (Fig. 2). Because NOC does not have an obvious RNA-binding motif (outside of its substrate-binding catalytic pocket), we presume that it recognizes its targets via binding to RBPs. Deadenylation by NOC may result in destabilization or translational silencing of the message. In either case, NOC’s activities would alter the protein composition of the cell. In the liver, this may help the clock shape the daily temporal profile in lipid storage or utilization.

Much still needs to be learned about the role of NOC in circadian posttranscriptional regulation and about the mechanism by which it functions. In particular, identification of NOC’s mRNA targets in the different tissues in which it is expressed will be key. However, the identification of this rhythmic deadenylase provides an entrée into increased understanding of how the clock controls the protein expression patterns that drive the many physiological rhythms necessary for normal homeostasis.

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