Rhythmic Expression of Nocturnin mRNA in Multiple Tissues of the Mouse

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Abstract

Background: Nocturnin was originally identified by differential display as a circadian clock regulated gene with high expression at night in photoreceptors of the African clawed frog, Xenopus laevis. Although encoding a novel protein, the nocturnin cDNA had strong sequence similarity with a C-terminal domain of the yeast transcription factor CCR4, and with mouse and human ESTs. Since its original identification others have cloned mouse and human homologues of nocturnin/CCR4, and we have cloned a full-length cDNA from mouse retina, along with partial cDNAs from human, cow and chicken. The goal of this study was to determine the temporal pattern of nocturnin mRNA expression in multiple tissues of the mouse.

Results: cDNA sequence analysis revealed a high degree of conservation among vertebrate nocturnin/CCR4 homologues along with a possible homologue in Drosophila. Northern analysis of mRNA in C3H/He and C57/B16 mice revealed that the mNoc gene is expressed in a broad range of tissues, with greatest abundance in liver, kidney and testis. mNoc is also expressed in multiple brain regions including suprachiasmatic nucleus and pineal gland. Furthermore, mNoc exhibits circadian rhythmicity of mRNA abundance with peak levels at the time of light offset in the retina, spleen, heart, kidney and liver.

Conclusion: The widespread expression and rhythmicity of mNoc mRNA parallels the widespread expression of other circadian clock genes in mammalian tissues, and suggests that nocturnin plays an important role in clock function or as a circadian clock effector.

Introduction

Circadian rhythms, synchronized to external environmental cycles such as day and night, occur in a broad range of physiological and behavioral processes. Endogenous oscillators or clocks capable of sustained oscillation through multiple cycles control such rhythms in the absence of external cues [1,2]. Molecular-genetic analysis of circadian rhythms in Drosophila, Neurospora and
more recently in vertebrate systems has led to the conclusion [3,4,5] that rhythms of gene expression are of central importance both in the sustained generation of rhythmicity (clock genes) and in the control of output pathways (clock controlled genes).

Recently, the search for components of the vertebrate circadian system has led to the identification of homologues of period [6,7,8,9,10,11,12] and timeless [13,14,15,16], originally characterized as central clock genes in Drosophila, as well as Clock and Bmal1 (Cycle), identified initially in the mouse [17,18,19,20,21]. In Drosophila CLOCK and BMAL1 regulate transcription of period (per) and timeless (tim) in a cycle in which the PER and TIM proteins dimerize, enter the nucleus, and negatively regulate their own transcription [22,23]. This pattern of rhythmic gene transcription appears to be of central importance to the clock mechanism. In addition, rhythmic regulation of the transcription of "clock controlled" genes such as tryptophan hydroxylase is important in the regulation of overt rhythms downstream of the clock [24,25].

Among vertebrates, circadian oscillators have been formally identified in the suprachiasmatic nucleus of the mammalian brain [26], the retina [27,28,29], and the pineal gland of non-mammalian vertebrates [30,31]. Each of these systems controls behavioral, physiological or neuroendocrine rhythms that are of physiological importance to the organism. However, it has recently become apparent that rhythmic gene expression occurs more broadly. For example, in Drosophila the circadian oscillator controlling behavioral rhythmicity can be localized to a small set of lateral neurons in the brain [32] while circadian transcription of the clock gene, period, occurs in multiple tissues and organs even when isolated to an in vitro setting [33]. The recent identification of period gene homologues in mammals has led to a similar finding of rhythmic expression in multiple tissues [7,8,10,11,12]. In one case, sustained rhythmicity has been demonstrated in tissue culture [34].

The nocturnin gene was discovered in a differential display screen for circadian gene expression using the retina of the African clawed frog, Xenopus laevis [35,36]. The gene encodes a protein with a leucine repeat domain and a domain homologous to the carbon catabolite repression 4 protein (CCR4), a transcription co-activator in yeast [37]. Analysis of the EST database also revealed human transcripts with extensive sequence similarity to the same domain in yeast CCR4 and NOCTURNIN [36]. CCR4 is thought to affect gene transcription through interactions with other proteins in the yeast transcriptional apparatus [37]. In Xenopus retina, nocturnin was found to exhibit high amplitude rhythmicity in which most, if not all, of the nighttime increase in mRNA could be accounted for as increased gene transcription [36].

Although the nocturnin gene appears to encode a potentially important component of the circadian regulatory system in the Xenopus eye, its position within or downstream of the circadian clock mechanism has not been determined. In addition, its importance in mammalian circadian regulation and in systems outside of the eye has not been evaluated. Here we report that a mouse homologue of nocturnin is expressed in a circadian pattern in multiple tissues including retina, spleen, kidney, heart and liver. Widespread rhythmic expression of mouse nocturnin (mNoc) parallels the pattern seen for other clock-related genes in the mouse, indicating that nocturnin is broadly associated with other circadian regulatory components.

Results
Homologues of Xenopus Nocturnin

Blast analysis of public databases reveals a large number of coding sequences with significant similarity to Xenopus nocturnin (xNoc). As we originally reported [36], XNOC protein is similar to a large, C-terminal domain of the transcriptional regulator, CCR4, but appears to be lacking in several regulatory domains critical to CCR4 function. Recently, however, mouse and human cDNAs encoding homologues (Fig. 1A) of the same domain of CCR4, but comparable in size to XNOC [38,39] have been reported (Accession number # AAD56547 and AAD56548). Furthermore, availability of the complete genome of Drosophila melanogaster [40] has revealed a coding sequence (AAF54601.1) with significant similarity to XNOC. In addition to these, we have recently added a complete coding sequence of mouse nocturnin cDNA from retina (mNoc) along with partial coding sequences for human (hNoc), bovine (bNoc), rat (rNoc) and chicken (cNoc).

Among the sequences illustrated in Figure 1, NOCTURNIN shows a high level of conservation throughout its coding sequence. As aligned, xNoc is 66% and 65% identical to HNOC and MNOC respectively. The aa identity drops to 36% when compared to DNOC. Among the vertebrate species this conservation is particularly striking beginning at aa 67. This methionine (not aligned in the Drosophila sequence) corresponds to the fourth codon of Xenopus [36] and mouse exon II [39]; the ATG at this site meets the Kozak [41] consensus for translation initiation in all three species and represents a possible alternative site of translation initiation. In the region beginning at the start of exon II XNOC is 78% identical to HNOC. The short coding sequence of XNOC exon I (22 aa) aligns poorly with a longer amino terminal region in the other species. In addition to the high sequence simi-
larity, *Xenopus* [36] and mouse [39] genes have a simple 3 exon structure with very similar boundaries of the second and third exons.

Previously, an unusual leucine zipper-like domain was identified in *Xenopus* nocturnin [36]. The third leucine in the *Xenopus* sequence is either a tyrosine (mouse and rat) or a phenylalanine (human, cow and chicken) in other vertebrates (see bar in Fig. 1). Furthermore, the alanine in position 4 of the second heptad is replaced by a proline in all five species. The latter proline is adjacent to a conserved proline identified earlier in XNOC (Fig. 1). These proline residues are not compatible with the coiled-coil structure characteristic of leucine zipper mo-

Figure 1
Comparison of the conceptual amino acid sequences of nocturnin homologues from chicken (cNoc), cow (bNoc), rat, (rNoc), *Xenopus* (xNoc), mouse (mNoc), human (hNoc) and Drosophila (dNoc). Sequences were analyzed using the Clustal W Alignment procedure. Gaps indicated with dots are inserted to achieve optimum alignment. Dark gray and light gray highlights indicate amino acid identities and similarities respectively. The horizontal bar marks the position of the heptad leucine repeat in *Xenopus* and the asterisks indicate the position of the leucines. The positions of introns 1 and 2 based on the *Xenopus* gene [36] are indicated by arrows. Note that both mouse [39] and human (from data in public databases of the National Library of Medicine) appear to have a similar gene structure based on 3 exons. The XNOC sequence is from GenBank accession number U74761, hNOC is from NP036250.1 [39], and dNOC is from AAF54601.1 [40]. Our complete mNoc cDNA from retina (accession number AF199491) has the same coding sequence as that reported earlier from liver [38,39]. The cNoc (AF199498), bNoc (AF199497), and rNoc (AF199495) partial sequences are from PCR amplified DNA segments.
mNoc is Expressed in Multiple Tissues of the Mouse

The principal goal of this study was to analyze mNoc mRNA expression. We used C3H/He mice because they are useful for studying clock activity based on rhythmic release of melatonin; C3H/He is one of the few mouse strains that synthesizes melatonin rhythmically [29,44]. In Northern analysis using single stranded probes generated from the mNoc 3' UTR or from exon II, we found that in contrast to our prior work in Xenopus, mNoc is expressed as a single mRNA of about 3 kb (Fig. 2). The only variations from this pattern was diffuse hybridization of the probe above the 3 kb position when gels contained higher levels of mNoc mRNA (Fig. 2, liver and kidney; Fig. 3, 4 and 5 at ZT 12) and diffuse hybridization below the 3 kb band specifically in spleen (Figs. 2, 5C); the latter may reflect RNA degradation.

mNoc is Rhythmically Expressed in Multiple Tissues

Northern analysis of retinal RNA shows that, as was the case in Xenopus, mNoc exhibits a rhythm of mRNA abundance. Peak expression occurs at the time of light...
offset (Fig. 3A). However, the amplitude of the rhythm is approximately 2 fold compared to the greater than 10 fold amplitude seen in *Xenopus* retina. Rhythmicity with a similar peak at ZT12 is also seen in heart (Fig. 3B), spleen (Fig. 3C), kidney (Fig. 3D), and liver (Fig. 4). The amplitudes of the rhythms in heart, spleen and kidney, as determined by phosphor imaging, reflect 2 to 5 fold changes between minimum and maximum. In contrast, the magnitude of the day-night difference in liver represents a nearly 30 fold change (see Fig. 4). Although the overall pattern of rhythmicity is similar in these tissues, baseline expression during the day is evident in retina, heart, and spleen and in part accounts for the lower amplitude in these tissues.

**Rhythms of mNoc mRNA Abundance are Circadian in Nature**

In order to investigate the endogenous rhythmicity of mNoc expression, C3H/He mice were maintained in constant darkness for 36 hours before sampling for rhythmic changes in DD. Samples were then taken in darkness at 6 time points referenced to the normal LD cycle in which they had been maintained (referred to as Zeitgeber Time). mNoc from all five tissues shows rhythmic changes in mRNA abundance (Figs. 4 and 5). Furthermore, liver tissue exhibits a high amplitude rhythm with virtually no mRNA detectable in the day-time as was the case in LD (Fig. 4B). The other four tissues all exhibit a higher level of daytime expression than in LD (Fig. 5). Unlike other tissues, spleen RNA exhibits a diffuse zone of hybridization centered at 1.0-1.2 kb, which may reflect RNA degradation.

mNoc mRNA appeared to reach higher levels at ZT12 in DD than in LD in all tissues except retina (compare Figs 3,4,5), suggesting that light may suppress mNoc mRNA level. This was particularly striking in liver where the ratio of mNoc/β-actin as determined by phosphor imaging was greater at ZT12 in DD than in LD (see Fig. 4D). This difference appears to be significant in that it was reproducible in an independent replication of the experiment in which LD and DD samples were analyzed on the same blot.
C3H/He and 129SV Mice Lack a Transposable IAP Element in the Nocturnin Gene

Laboratory strains of mice are heterogeneous in the presence of a transposable intracisternal A-particle (IAP) element in the nocturnin/CCR4 gene. During the course of our work on the mNoc cDNA it was reported [38,39] that a transposable IAP of viral origin, present in about 1000 copies throughout the mouse genome, is found in the first intron of the mNoc/CCR4 gene. In DBA/2, BALB/c, C57Bl/6 and C57Bl/10 mice, transcriptional read through from the IAP insert to the mNoc/CCR4 open reading frame resulted in hybrid transcripts (3, 6 and 10 kb) whose abundance increased in aging mice. Apparently, insertion of the IAP element in the first intron occurred relatively recently because the insert was found to be lacking in some strains of mice [39]. We confirmed the lack of an IAP element in 129/SV and C3H/He (the strain used in the rhythmic analysis above) mice through

Figure 4
mNoc mRNA in liver exhibits a high amplitude rhythm with peak expression at ZT12 in both LD (A and C) and DD (B and D). In LD samples were taken at 6 hour intervals as in Figure 4. Samples in DD were taken at Zeitgeber Times (ZT) 0 (24), 4, 8, 12, 16 and 20 referenced to the LD cycle immediately before DD treatment. Mice were in DD for 36 hours before beginning collections. The rhythmic changes illustrated are representative of three replicates for LD and two for DD. Phosphor imaging was used as in Figure 4E to quantitate mNoc mRNA levels (C and D). Note that the amplitudes of the rhythms are much higher in liver than for other tissues.
a combination of genomic PCR and partial sequencing of a genomic clone. As shown in Figure 6, genomic PCR using primers from sites in the intron adjacent to the IAP position revealed only intronic sequence in C3H/He and 129/SV mice. This was further confirmed for 129/SV mice by the lack of an IAP element in genomic sequence from this region as well (data not shown). Although the IAP element is absent in these two strains of mice, we confirmed the presence of the IAP sequence by PCR in BALB/c (Fig. 6B) and C57/Bl6 (data not shown) mice as reported [39].

**Rhythmic Expression of mNoc in BALB/c Mice**

We tested the hypothesis that the IAP element in Intron I of BALB/c mice ([14,36] see Fig. 6) would disrupt rhythmic expression of mNoc. Six week old BALB/c mice were kept in our animal facilities for two weeks and liver and kidney tissues were obtained for Northern analysis at 6 hour intervals through a light-dark cycle. As shown in Figure 7, mNoc in BALB/c mouse liver and kidney clearly exhibits rhythmicity similar to that seen in C3H/He mice with a prominent mRNA band at approximately 3 kb. Less abundant larger bands are also seen above 4 kb (liver and kidney) and 8 kb (liver only). Although larger mRNA bands have been reported to reflect hybrid transcripts including components of the IAP element in aging BALB/c mice, it is unclear whether this would explain the larger bands in Figure 7. The bands in Figure 7 are smaller than the 6 and 10 kb bands reported in old mice [38,39] and follow a rhythmic pattern similar to that of the 3 kb band. The larger bands could reflect splicing intermediates that are seen only during the period of maximal transcription of mNoc. Although it is possible that altered transcription of mNoc as a consequence of an interaction between aging and the IAP insert may alter the rhythmic pattern of expression [39], our data clearly indicate that the mere presence of the IAP element in mice 8 weeks of age has little or no impact on rhythmic expression. Comparable results have been obtained using C57/Bl6 and CBA/J mice (data not shown).
**C3H/He and 129/SV mice lack an intracisternal A-particle (IAP) insert in the first intron of the nocturnin/CCR4 gene.**

**A.** Diagram showing the results of partial sequencing of a nocturnin genomic clone derived from a 129/SV bacterial artificial chromosome (BAC) library. Green boxes identify regions of medium repetitive sequences in the mouse genome. The blue arrows represent mNoc coding regions corresponding to Xenopus exons II and III separated by an intron. The IAP element identified in BALB/c mice (bar above) was expected between bp 6583 and 6584 in our BAC sequence based on published data [39] but was lacking in this BAC sequence. F1, F2 and R1 are the positions of forward and reverse primers used for genomic PCR. Note that there are two potential polyadenylation sites in the 3' UTR 650 base pairs apart. The 3' UTR probe used in our northern analysis lies between the two potential polyadenylation sites, and hybridizes to the same mRNA band as the probe derived from Exon II. This suggests that only the most 3' site is used.

**B.** Genomic PCR with primers from the BAC clone flanking the IAP site (labeled as F1 and R1 in A) resulted in a 107 bp band in C3H/He and 129SV mice that was lacking in BALB/c mice. Pairing the R1 primer from the BAC clone with primer F2 derived from the IAP sequence (see A) resulted in a 1500 bp band in BALB/c mice but not 129SV or C3H/He. DNA sequencing revealed the expected sequence from the BAC clone for the 107 bp band and the expected sequence from the IAP insert for the 1500 bp band. The lanes labeled 412D6 are control PCR reactions using the 129/SV BAC as template. Note that the absence of ≈ 5 Kb band for BALB/c mice with primers F1 and R1 is due to the inefficiency of Taq polymerase for large products; we have separately obtained the full IAP insert in BALB/c mice using a long PCR procedure. DNA size markers are included in the two outer lanes.
Expression of mNoc in the Brain

As shown in Figure 2, mNoc mRNA is expressed in tissue from the mid-brain, which contains the hypothalamic nucleus. In several attempts at temporal Northern analysis in LD using samples excised from the midbrain of C3H/He and C57/Bl6 mice, we saw hints of low amplitude rhythmicity, but the results were variable and could have resulted from sampling error (data not shown). We, therefore, examined brain expression of mNoc mRNA further using in situ hybridization of tissue from C57/Bl6 mice fixed at ZT 4, ZT 12, and ZT 20. mNoc transcripts were detected (Fig. 8) in the suprachiasmatic nucleus (SCN), the ventral hypothalamic nucleus, arcuate nucleus (Arc), the piriform cortex (Pir), the hippocampus (Hip), the cerebellum, the subiculum, the internal granule layer of the olfactory bulbs and the pineal gland. Although we saw variability at different sample times (Table 1) in the intensity of the hybridization in several brain regions, the observations were of a qualitative nature and the magnitude of the changes was not great. Although the in situ hybridization data suggest low amplitude rhythmicity in these brain regions (including the SCN), we have not detected rhythmic expression in the brain comparable to the high amplitude rhythmicity detected in peripheral tissues.

Table 1: Semi-quantitative analysis of mNoexpression in different brain regions at three times of day. (++ = Strong hybridization; + = Weak hybridization; - = no hybridization).

| Brain Region              | ZT4 | ZT12 | ZT20 |
|---------------------------|-----|------|------|
| Olfactory Bulb            |     |      |      |
| Internal Granule Layer    | ++  | ++   | ++   |
| Lateral olfactory tract   | -   | -    | -    |
| Piriform cortex           | ++  | +    | -    |
| Hippocampus               | ++  | ++   | +    |
| Hypothalamus              |     |      |      |
| Suprachiasmatic nucleus   | +   | +    | -    |
| Arcuate nucleus           | +   | +    | -    |
| Ventromedial hypothalamic |     |      |      |
| Nucleus                   | +   | +    | -    |
| Subiculum                 | +   | +    | +    |
| Cerebellum                | ++  | ++   | +    |
| Pineal Gland              | -   | -    | +    |

Figure 7

mNoc mRNA is expressed rhythmically in 8 week old BALB/c mouse liver (A) and kidney (B) in a light dark cycle (LD). Tissues for RNA extraction were collected at Zeitgeber Times (ZT) 0 (24), 6, 12 and 18 with lights on at ZT 0 and off at ZT12. Images of the methylene blue stained 28S and 18S rRNA bands on the same blot are shown below as loading controls.
Discussion
Our principal findings are that both the structure of the putative NOCTURNIN protein and circadian expression of its mRNA are conserved in the mouse. In addition, partial cDNA sequences and database analysis reveals $xNoc$ homologues in Drosophila, human, rat, cow, and chicken. $xNoc$ was originally identified as the product of a differential display screen for circadian clock-regulated genes [35,36] in the retina of the African clawed frog, a system known to exhibit circadian clock activity in an in
**The widespread circadian expression of mNoc mRNA in multiple tissues of the mouse parallels that of the Drosophila central "clock gene", period, which was recently characterized in mammals. The period gene is rhythmically expressed in multiple tissues as well as in the central "clock" controlling behavior in both Drosophila [33] and mouse [7,8,10,11,12]. At present we do not know if nocturnin plays a role as a central component of the circadian clock mechanism or as a "clock controlled" gene, perhaps coupling clock activity to an unidentified physiological rhythm. However, widespread expression and rhythmic regulation of mNoc argues against a limited role in rhythmic physiology of the retina or in the regulation of its melatonin output rhythm suggested by the earlier work on the Xenopus eye. It seems more likely that nocturnin is coupled to circadian function in a general way as either a central clock component or as a downstream effector.

During the course of our work on mNoc, it was reported [38,39] that a transposable intracisternal A-particle (IAP) element of viral origin is found in the first intron of the mouse nocturnin/CCR4 gene. Furthermore, it was reported that in DBA/2, BALB/c, C57Bl/6 and C57Bl/10 mice, transcriptional read through from the IAP transcriptional start site to the nocturnin/CCR4 open reading frame resulted in hybrid mRNA transcripts whose
abundance increased in parallel in aging mice. This report raised the immediate concern that disrupted mNoc transcription might modify its rhythmic expression pattern and function. However, insertion of the transposable IAP in the nocturnin/CCR4 gene was apparently a recent event, occurring after the origin of modern mouse strains because some mouse strains lack the insert [39]. Our genomic sequencing and PCR experiments confirm this finding by demonstrating that the IAP element is present in Balb/c and C57/B16 mice but lacking in 129/SV and C3H/He mice. Thus, the rhythmic expression of mNoc as a single mRNA species in C3H/He mice appears to reflect the wildtype condition for this gene.

The IAP insert appears to strongly affect the expression of mNoc mRNA in aging mice. The multiple hybrid forms of mNoc mRNA [38] along with the recent report of an absence of rhythmic expression [39] of mNoc mRNA in mice containing the IAP element have raised the possibility that differences among mouse strains could provide a basis for understanding nocturnin function. It is well known that strains of inbred mice have different rhythmic phenotypes. Perhaps the best understood is the lack of the ability to produce melatonin in some strains such as BALB/c and C57/B16 and the production of normal rhythms of melatonin in others such as the C3H/He and CBA [44,46,47,48,49]. Recently, we initiated studies directed at analysis of mNoc expression in BALB/c mice with the goal of determining if the IAP insertion has a direct consequence on rhythmic mNoc expression. Our analysis shows that the presence of the IAP element has no impact on mNoc expression or its rhythmicity in mice up to 8 weeks of age. Although it is possible that altered expression of the mNoc gene during the process of aging may affect the rhythmic phenotype, our data indicate that the IAP insert itself cannot be regarded as a specific insertional mutation with direct consequences on rhythmicity.

Although nocturnin was originally identified as a rhythmic gene product in photoreceptors, the most striking rhythmity identified in the mouse is in the peripheral tissues such as liver and kidney. In parallel with the findings of this study we recently identified nocturnin as a rhythmic gene product in rat liver and kidney based gene array analysis of over 9000 rat Unigenes (Kita, et al., unpublished). Interestingly, in the latter study rNoc was identified among a group of clock-regulated genes that included Per1, Per2, Per3, Bmal1, and D-binding protein (DBP). Independent clock driven pathways may be critical in the function of many tissues and organs as suggested by the widespread expression of clock genes in peripheral tissues [7,8,10,11,12]. Support for this conclusion comes from the recent finding that circadian oscillation of gene expression in the liver is entrained by food intake independently of the central oscillator in the brain [50]. An understanding of the rhythmic function of nocturnin may come from analysis of its role in rhythmic physiology of the liver and kidney.

Materials and Methods

Animals and tissue collection
C3H/He mice, wild type (+/+) at the rd locus, were originally obtained from Dr. Michael Menaker at University of Virginia and then maintained as a breeding colony in ventilated environmental compartments within a temperature-controlled animal facility (24 -25°C) on a 12 hour light:12 hour dark cycle (LD), except as noted. BALB/c, CBA/J and C57/B16 mice were purchased from Charles River Laboratories (Wilmington, MA) or Jackson Laboratories and maintained under similar conditions. Experimental protocols were approved by the Institutional Animal Care and Use Committee and follow all federal guidelines. Mice were sacrificed by cervical dislocation following exposure to carbon dioxide or an overdose halothane anesthesia. Tissues for RNA extraction in LD were collected at ZT0, ZT6, ZT12 and ZT18 in light (standard room fluorescent light) or dim red light (Kodak Watten #2, filters). Those for constant dark (DD) experiments were collected in dim red light at ZT0, ZT4, ZT8, ZT12, ZT16 and ZT20 referenced to the LD cycle immediately before DD treatment. Bovine, chicken, rat (Sprague Dawley obtained from the ARC) and human retinal tissue (obtained from the Eye Research Institute at Medical College of Wisconsin) were immediately frozen on dry ice after dissection and stored at -80°C.

Total RNA and Genomic DNA isolation
Total RNA was extracted by the TRIZOL reagent protocol (GIBCO/BRL, Rockville, MD), and then dissolved in DEPC-treated water before storage at -80°C. QIAamp Tissue Kits (Qiagen Inc., Santa Clara, CA) were used for genomic DNA extraction from liver tissues according to the kit protocol.

cDNA Library Screening and DNA Sequencing
Mouse nocturnin cDNA (mNoc) clones were obtained by screening a mouse BALB/c retinal cDNA library from ATCC (ATCC# 77448, Rockville, MD; reference [51]) using the standard protocol published in Sambrook, et al, 1989 [52] with probes from a human EST (T87026) purchased from Research Genetics, Incorporated (Huntsville, AL). mNoc clones were custom sequenced by Sequtech Corporation (Mountain View, CA). The human EST (T87026) and additional human clones obtained by PCR were sequenced manually using T7 Sequenase 2.0 DNA sequencing kit (Amersham, Piscataway, NJ) or using a BigDye terminator cycle sequencing kit on an ABI Prism 310 capillary sequencer (PE Applied
Biosystems, Foster City, CA). MacVector software was used for sequence analysis in this study.

**Genomic Library Screening and Analysis**

*mNoc* bacterial artificial chromosome (BAC) clones were obtained from Research Genetics, Incorporated (Huntsville, AL) by custom screening of a 129/SV BAC library with the "whole cell PCR" protocol. BAC DNA was isolated by a Qiagen Plasmid Maxi Kit and sequenced using the ABI Prism 310. For the genomic PCR in Figure 6 the forward primer F1 (5'-AGTGACTGTCCCTTCCTGT-3') is located upstream (5') and the reverse primer, R1 (5'-AACACAGTGAGAGCCTGCTT-3') is located downstream (3') of an intracisternal A-particle (IAP) element (reference [39]) identified in the nocturnin/CCR4 gene. The forward primer F2 (5'-TGATGTCCAGGGCGTCAG-3') is located in the IAP element itself [39]. The sequences for F1 and R1 are based on sequences from the BAC clone characterized in Figure 6A while the sequence of F2 is from the IAP element (reference [39]). These three primers were used for genomic PCR with Taq DNA polymerase (Promega). All of the resulting PCR products were cloned into pCRII-TOPO (Invitrogen, Carlsbad, CA) and sequenced as above.

**Probe Preparation and Labeling**

Single strand PCR probes for Northern hybridization representing 553 bp of 3' UTR or Exon II of *mNoc* were generated using a modification of the single-strand DNA protocol of Bednarzuk, et al. [53], including 32P-dCTP (40 mCi/ml; NEN Life Science Products, Boston, MA) in the reaction mixture. The primers for the 3'UTR probe were 5'-AACATCGTCAAAGCTCTC-3' (bp 1557-1575 of the mNoc cDNA, forward) and 5'-GGCTGGAAGGTGTCAAAG-3' (bp 355-374, forward) and 5'-GGCTGGAAGGTCAAGACG-3' (bp741-759, reverse). Random primed probes were prepared using the Random Primers DNA Labeling Kit (GIBCO/BRL, Rockville, MD). Radioactive probes were purified through NucTrap gel filtration columns (Stratagene, La Jolla, CA).

**Northern Blot Analysis**

Ten µg (or less as specified) of each RNA sample was separated on 1.0% formaldehyde-agarose gels using standard procedures [35]. Northern blot analysis was carried out according to QuikHyb hybridization solution protocol (Stratagene, La Jolla, CA). Nylon membranes were stripped by washing twice for 10 min in boiling 0.01X SSPE (0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA, 0.5% SDS) and rehybridized with probes made from mouse β-actin cDNA [54]. Hybridization signals were quantitated using a Storm PhosphorImager and ImageQuant software (Molecular Dynamics) using a previously described method [36].

**5' RACE and PCR reactions**

Total RNA used as a template in 5'-RACE and RT-PCR was treated with RNase-free DNase I (Promega, Madison, WI) and subsequently phenol-chloroform extracted. RNasin Ribonuclease Inhibitor (Promega) was used in both 5'-RACE and RT-PCR reactions. 5'-RACE was performed according to kit protocol (GIBCO/BRL, Rockville, MD). The reverse Transcription System (Promega) coupled with Taq DNA Polymerase (Promega) was used for RT-PCR.

Degenerative PCR was carried out using Taq DNA Polymerase (Promega) with 5'-GATGGGAAC(A/G)GACCAG(C/T)(A/C)GAC-3' and 5'-GC(G/C)AG(A/G)ATGTTCCAATGAT(C/G)AC-3' as forward and reverse primers respectively. The resulting PCR products were cloned into pCRII-TOPO and sequenced with an ABI Prism 310 sequencer.

**Brain In Situ Hybridization**

C57/B6 mice were decapitated following an overdose of halothane anesthesia. The brain was removed, frozen on dry ice and stored at -80° until sectioning. In situ hybridization followed the protocol of Fukuhara, et al. [55]. α-32P UTP (1250 Ci/mmol; NEN Life Science Products, Boston, MA) labeled probes were obtained by in vitro transcription. A mouse nocturnin cDNA fragment (450 bp) cloned into the pBluescript KS (+) vector (Stratagene) was linearized with Xhol or XbaI for antisense or sense probes, and radiolabeled using T7 or T3 RNA polymerase respectively. Serial coronal cryostat sections (20 µm thick) were hybridized overnight at 55°C and washed at 57°C. Slides were exposed to Kodak Biomax film for 6 days at room temperature.

**GenBank Accession Numbers**

Sequences completed for this work have been placed in GenBank. Newly assigned GenBank Accession numbers for these sequences are AF199491 (mNoc), AF199492 (hNoc, genomic fragment), AF199493 (hNoc, RT-PCR product), AF199494 (hNoc, EST T87026), AF199495 (rNoc, 5' RACE product), AF199496 (rNoc, RT-PCR product), AF199497 (cNoc) and AF199498 (cNoc).

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