The circadian gene Clock is required for the correct early expression of the head specific gene Otx2

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ABSTRACT The circadian cycle is a universal molecular mechanism for imposing cyclical control on cellular processes. Here we have examined the role of one of the crucial circadian genes, Clock, in early Xenopus development. We show that a dominant negative version of Clock can block the function of the endogenous Clock gene. Doing so during early development reduces Otx2 expression in a highly specific manner and results in anterior defects. Together with previous work (Green et al. (2001) Mech. Dev. 105-110), these results suggest that a positive regulatory loop exists between Clock and Otx2.

KEY WORDS: Circadian cycle, Clock, head development, Otx2

Introduction

The circadian cycle is the regular, periodic oscillation of cellular and metabolic activity in anticipation of daily environmental changes. Although the existence of such cycles have been known for a long time, it is only in the last few years that the underlying molecular processes have been elucidated (reviewed by Green, 1998, Dunlap, 1999 and Lakin-Thomas, 2000; Cermakian and Sassone-Corsi, 2000). The daily oscillations depend upon the interactions of a relatively small number of components that have been highly conserved in evolution.

In mammals, a complex formed by the transcription factors CLOCK (CLK) and BMAL1 activates the expression of the Period (Per) and Cryptochrome (Cry) genes (Antoch et al., 1997; King et al., 1997; Tei et al., 1997; Gekakis et al., 1998; Hogenesch et al., 1998). As PER protein is made, the caesin kinase I (CKI) protein binds to PER, masking its nuclear localization signal and retaining it in the cytoplasm in a phosphorylation dependent manner (Vielhaber et al., 2000). After a delay, a complex between PER, CRY and CKI enters the nucleus, where CRY inhibits the CLK:BMAL1 complex and the transcriptional activation of Per and Cry genes (Shearman et al., 2000). The level of PER and CRY proteins falls until the CLK:BMAL1 complex is no longer inhibited, at which point transcription of Per and Cry increases again. One of the per genes (per2) has also been implicated in a positive role, as the PER2 protein promotes activation of the Bmal1 gene (Shearman et al., 2000). These events cause the level of CRY, PER and BMAL1 proteins to cycle on a roughly twenty four hour basis, the latter having an opposing phase to its two target genes (Shearman et al., 1999). Very little is known about how this timing information is communicated to the many outputs of the clock, although in some cases downstream rhythmic genes have been shown to be under the direct transcriptional control of CLOCK and BMAL1 (Jin et al., 1999).

The primary area for circadian control in adult vertebrates is the suprachiasmatic nuclei (SCN) in the brain (reviewed by Weaver, 1998). These neurons show very strong circadian activity, and can relay circadian information to other parts of the nervous system and hence also a number of target organs (Sakamoto et al., 1998; Oishi et al., 1998; Yamazaki et al., 2000; Yagita et al., 2001). This applies also to embryonic cells where the SCN has not yet formed. In particular, zebrafish embryos inherit circadian cycle information through the egg and maintain the same phase from this point throughout development (Deluanay et al., 2000).

Despite these advances, relatively little is known about the control and function of the Clock gene in early development. In a recent study, we examined the early expression of the Clock gene in the frog (Xenopus laevis, Green et al., 2001). The Xenopus homologue of Clock (Xclk) is very similar to the mouse and human genes and is expressed in the retinal photoreceptor cells at a constant level (Zhu et al., 2000). Like the murine clock (Mclk) gene, Xclk does not seem to undergo cyclical changes in expression early on in development (Tei et al., 1997; Shearman et al., 1999; Green et al., 2001). Its expression is not controlled by the suprachiasmatic nuclei (SCN) and does not regulate the expression of other clock genes.

Abbreviations used in this paper: CKI, caesin kinase I; CLK, clock gene; GFP, green fluorescent protein; Per, period gene.

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expression is initially restricted to the anterior neural plate and then subsequently expands along the whole neural tube. Xclk expression is an early response to neural induction, and is strongly upregulated by the homeodomain containing transcription factor Otx2 early in gastrulation (Green et al., 2001). This regulation is direct (i.e. it is independent of further rounds of transcription and translation; Green et al., 2001).

The Otx2 gene and its homologues are highly conserved both in sequence and function, and are expressed exclusively in the head, where they are required for the development of the forebrain and midbrain structures at a very early stage in development. Mice lacking Otx2 function do not form a recognizable forebrain (Ang et al., 1996) and otd-deficient Drosophila embryos lack the most anterior portion of the head (Finkelstein et al., 1990; Finkelstein and Perrimon, 1990). Additionally, Otx2 can substitute for otd function in Drosophila and otd can substitute for Otx2 function in mice (reviewed by Klein and Li, 1999; Hirth and Reichtert, 1999). These remarkable findings reflect the very high degree of functional conservation within the Otx gene family.

Here we describe the use of a dominant negative Clock construct that blocks transcriptional activation of Clock target genes and hence interferes with circadian activity (Hayasaka et al., 2002). We show that, in addition to being an upstream regulator of Xclk, Otx2 is also a target of regulation by Xclk. This suggests the existence of a positive feedback regulatory loop between Xclk and Otx2. We show that blocking Xclk function early on in development interferes with normal Otx2 expression and causes abnormal development of the head.

Results

CLΔQ-GFP blocks the Activation of a Clock Responsive Promoter in the Early Embryo

The action of the endogenous Xenopus Clock (Xclk) gene can be blocked by a deletion construct of Xclk which removes the C-terminal Q rich domain, replacing it instead with Green Fluorescent Protein (GFP). The Q-rich domain is required for transcriptional activation (Gekakis et al., 1998), hence the resulting construct (CLΔQ-GFP) can still bind the E-box consensus sequence and BMAL1 protein, but can no longer activate transcription. Xclk target genes are expressed by the endogenous XCLK protein (Hayasaka et al., 2002).

In order to test whether CLΔQ-GFP could block endogenous Xclk function in the early embryo, we used a CLK responsive reporter construct, consisting of multiple copies of the E-box consensus region upstream of the luciferase (luc) open reading frame (Fig. 1A). This reporter construct was injected into fertilised eggs either alone, or with varying amounts of CLΔQ-GFP RNA and Xclk RNA. The embryos were harvested at the early neurula stage (13) and the amount of luciferase activity determined by an enzymatic assay (Fig. 1B). It is apparent from this data that CLΔQ-GFP almost totally prevents the activation of the E-box/luc reporter when 400pg of RNA are injected. Furthermore, this inhibition can be reversed when an excess (1000pg) of unmodified Xclk RNA is co-injected with CLΔQ-GFP (Fig. 1B).

CLΔQ-GFP Expression causes a Specific Anterior Defect which can be rescued by Xclk Over-Expression

In order to examine the effect of inhibiting Clock activity during early development, we injected fertilised eggs with CLΔQ-GFP RNA, either alone or in combination with Xclk RNA. Development was then allowed to proceed to the tailbud stage (Fig. 2).

It is apparent that expression of CLΔQ-GFP causes a slight but distinct change in the morphology of the head region (Fig. 2B). Compared to the untreated controls it is distinctly smaller, and the eyes appear to be abnormally formed. In order to demonstrate that this effect is due to the inhibition of Xclk and is not a non-specific effect related to the injection of RNA, we attempted to rescue the observed phenotype by co-injecting Xclk RNA (Fig. 2C). This restored the normal head morphology in 42% of cases (Fig. 2D).

CLΔQ-GFP specifically inhibits Otx2 Expression

We wanted to establish whether the observed changes in morphology when Xclk is inhibited relates to specific changes in gene expression. To this end, we extracted RNA from embryos in the previous experiment (see 3.2) at the neurula stage (15), and examined the expression of a number of marker genes expressed in distinct regions along the anterior to posterior (anteroposterior) axis by RT-PCR (Fig. 3A). It is apparent from this data that Otx2 is specifically down-regulated by CLΔQ-GFP, whilst its normal expression is restored by co-injecting Xclk RNA.

We examined Otx2 and Hoxb-4 expression in these embryos by in situ hybridisation with antisense Otx2 and Hoxb-4 probes (Fig. 3 B-D). Otx2 expression is strongly in the eye and midbrain regions of the untreated control.
embryos at this stage (Fig. 3B), and its expression is reduced by CLΔQ-GFP injection (Fig. 3C). A significant restoration of Otx2 expression is apparent when Xclk is co-injected with CLΔQ-GFP (Fig. 3D). The expression of the more posteriorly expressed gene Hoxb-4 (posterior hindbrain and spinal cord) is not affected by these treatments.

**Xclk indirectly up-regulates Otx2 in CLΔQ-GFP Injected Embryos**

It is apparent that Xclk can upregulate Otx2 expression in CLΔQ-GFP injected embryos. We wanted to determine whether this upregulation is direct (i.e. independent of further translation) or indirect. For this purpose we used a fusion between Xclk and the human glucocorticoid receptor (CLK-GR). The glucocorticoid receptor binds heat shock proteins, preventing it from entering the nucleus. This steric hindrance of nuclear entry is relieved by ligand binding, in this case the glucocorticoid analogue dexamethasone (dex), which by itself has no discernible effects on Xenopus development (Gammill and Sive, 1997). Hence the CLK-GR construct confers dex dependence on the activity of Xclk.

We injected fertilised eggs with CLK-GR RNA either alone or in combination with CLΔQ-GFP RNA. Embryos were allowed to develop to the mid-neurula stage and then treated with dex in the presence or absence of cycloheximide (chx), which blocks protein synthesis. We examined the expression of Otx2 by RT-PCR of RNA subsequently extracted from these embryos (Fig. 4). It is apparent from this data that the activation of Otx2 transcription by Xclk in CLΔQ-GFP injected embryos is dependant on protein synthesis (i.e. it is indirect).

**Discussion**

Here we have shown that, along with its established role in circadian timing, Clock also has a role in early development. Specifically, blocking Clock function using a dominant negative construct disrupts anterior development, resulting in an abnormally shaped, hypotrophic head with a smaller midbrain and eye. The principal change in gene expression
associated with this is a reduction in the amount of Otx2 RNA, a gene known to play a vital part in the specification and development of the head.

We have also shown that this affect on Otx2 expression is specific to Clock activity, as normal development can be rescued by over expressing Clock. This rescue is associated with restored levels of Otx2 RNA, indicating that Clock can up-regulate or at least maintain Otx2 expression. The regulation of Otx2 by Clock is indirect (i.e. it requires an intermediate step requiring protein translation), and thus Clock must activate at least one more gene upstream of Otx2 and there may of course be others. In addition, it should be noted that although changes in Otx2 expression and in the structures in which it is expressed are the major changes observed when Clock activity is inhibited, there may be other, more subtle changes. Previous work has shown that Clock is in fact directly upregulated by Otx2 (Green et al., 2001). Thus there seems to be a regulatory loop involving positive feed back between the two genes (Fig. 5).

A number of recent studies have shown that various isoforms of CKI can bind to and phosphorylate mPER1 protein, retaining it in the cytoplasm and delaying its nuclear entry. In Drosophila, PER protein is phosphorylated by the Doubletime protein kinase, similar to the phosphorylation of PER by CKI in mammals. This phosphorylation destabilizes PER until it heterodimerizes with the TIM protein. This complex can then enter the nucleus where PER inhibits clock-mediated transcription. GSK-3 phosphorylates the TIMELESS protein, promoting its dimerisation with the PERIOD protein and resulting in the inhibition of CLOCK. GSK-3 is also a component of the wnt signaling pathway, but, unlike dvl, it actes negatively (reviewed by Cadigan and Nusse, 1997). Hence there is a further functional correlation, as GSK-3 acts indirectly to inhibit both clock and wnt signaling. We note however that the putative mammalian homologue of the tim gene does not seem to be involved in the circadian clock (Zylka et al., 1998; Kume et al., 1999; Gotter et al., 2000), but does seem to have an important function in early development (Gotter et al., 2000).

From the proceeding discussion then, it is apparent that there are multiple interactions between the circadian cycle and wnt signaling, mediated at least in part by Otx2 and CKI (Fig. 5). In light of this, it is hardly surprising that the circadian pathway is affected by early developmental events and that these events in turn affect the establishment of circadian timing. Further studies may help to elucidate the functional significance of these interactions.

**Materials and Methods**

**RNA Extraction and RT-PCR**

Total RNA was extracted from whole embryos using the QuickPrep Total RNA extraction kit (Amersham Pharmacia Biotech Inc.). 3 µg of RNA was used in subsequent reverse transcription reactions. This was mixed with a poly T 15 oligo to 5 µg/ml and heated to 75°C for 5 minutes. After cooling on ice, the following additional reagents were added: dNTPs to 0.4 mM, RNase OUT (Promega) to 1.6 U/µl, Moloney Murine Leukemia Virus

**Fig. 4. (Left) RT-PCR analysis of Otx2 and ef1α expression in neurula stage embryos.** Fertilised eggs were injected with either CLA-Q-GFP RNA, CLK-GR RNA, or both, and allowed to develop to the neurula stage. NIC, non-injected control. Before RNA extraction the embryos were treated with dexamethasone (dex) and cycloheximide (chx), either alone or in combination, as shown. ef1α was included as a loading control.

**Fig. 5. (Right) Putative interactions between the circadian cycle and the wnt signal transduction pathway.** The blue arrows indicate positive interactions and the red lines indicate inhibitory interactions. The boxed components are proteins and those in ovals are genetic loci. PER, Period; CKI, Caesin Kinase I; DVL, Dishevelled.
Reverse Transcriptase (M-MLVrT) RunaseH- point mutant (Promega) to 8 U/µl and the appropriate buffer (supplied by the manufacturer) to x1 concentration. The mixture was incubated for one hour at 37°C, heated to 70°C for two minutes and cooled on ice.

PCR reactions were all performed in a total volume of 40 µl. For each we used 1 µl of the M-MLVrT reaction (as described above), 0.2 nmols each of primer and 20 µl of Redimix pre-mixed PCR components (Sigma). All reactions were cycled at 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 60 seconds. 30 cycles were used for all primer sets except those for ef1α, for which 23 cycles were used. The sequences of the primer pairs can be found on the internet at:

http://www.sghms.ac.uk/depts/anatomy/pages/richhmpg.htm/

Embryo Culture and Microinjection
These were performed as described (Sive et al., 2000).

Luciferase Assays
These were performed as described previously (Morgan et al., 1999a), using 200 pg of linearised E-box-luc reporter construct.

Transcription of Capped mRNA for Microinjection
ClkGR and CLA-Q-GFP Expression Constructs
For ClkGR, the full length Xclk reading frame (accession number AF227985) was amplified from the Xclk cDNA cloned in pBlueScript (Zhu et al., 2000). This PCR product had XhoI and BamHI linkers, allowing it to be ligated into vector CS2+ containing the human GR ligand binding domain (Morgan et al., 1999b). The resulting construct, ClkGRCS2+, was a Xclk/GF fusion (the two sequences being in frame with each other). For CLA-Q-GFP (Hayasaka et al., 2002; kindly provided by C. B. Green), the first 1629 nucleotides of the Xclk reading frame were amplified by PCR and ligated into vector pEGFP-1 (Clontech). This construct lacks the poly-Q transcriptional activation region of Xclk, which has been replaced with the GFP encoding portion of the pEGFP-1 vector. The resulting CLA-Q-GFP reading frame together with the SV40 poly A region from pEGFP-1 was then amplified by PCR and the resulting product was cloned into pGEMT-easy (Promega) to give construct CLA-Q-GFPpGT.

References


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