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

RICHARD MORGAN*

Department of Anatomy and Developmental Biology, St. George's Hospital Medical School, London,UK

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; Zylka *et al.,* 1998). Additionally, many other cells in the adult also express the molecular components of the circadian cycle, and continue to cycle in the absence of control by the SCN (Besharse and Iuvone, 1983; Cahill and Besharse, 1993; Balsalobre *et al.,* 1998; Whitmore *et al.,* 1998; Zylka *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

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

***Address correspondence to:** Dr. Richard Morgan. Department of Anatomy and Developmental Biology, St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, U.K. Fax: +44-208-725-3326. e-mail: rmorgan@sghms.ac.uk.

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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 (Fig. 1A). It thus acts in a dominate negative fashion, interfering with the activation of *Xclk* target genes 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).

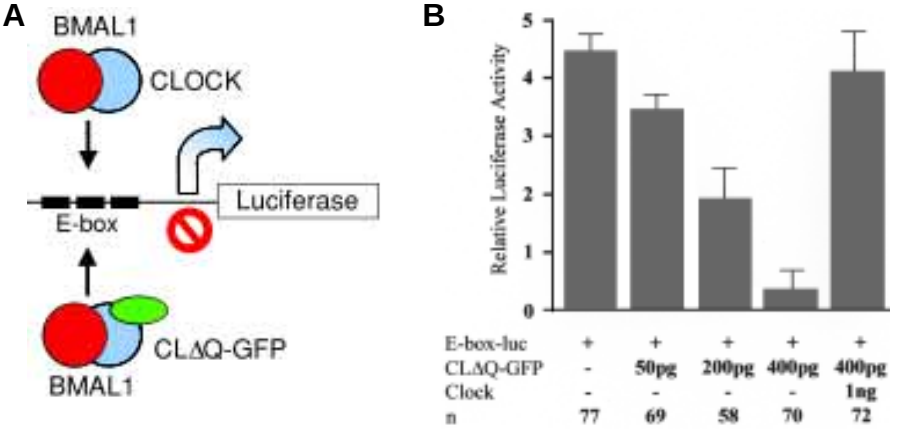

Fig. 1. CLΔQ-GFP acts as a dominant negative inhibitor of endogenous *Xclk* activity. (A) *The CLOCK/BMAL1 dimer binds the E-box consensus sequence in the reporter construct and activates transcription of the luciferase reporter gene. CLΔQ-GFP can still bind BMAL1 and the heterodimer can still bind the E-box sequence, although without activating transcription. It thus acts in a dominate negative fashion.* **(B)** *Reporter gene activity in embryos from untreated eggs or eggs injected with* CLΔQ-GFP *and / or* Clk *RNA as shown. The error bars shown are the standard deviations from the mean. The numbers in each sample are shown in the figure (n).*

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 downregulated 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* is expressed strongly in the eye and midbrain regions of the untreated control

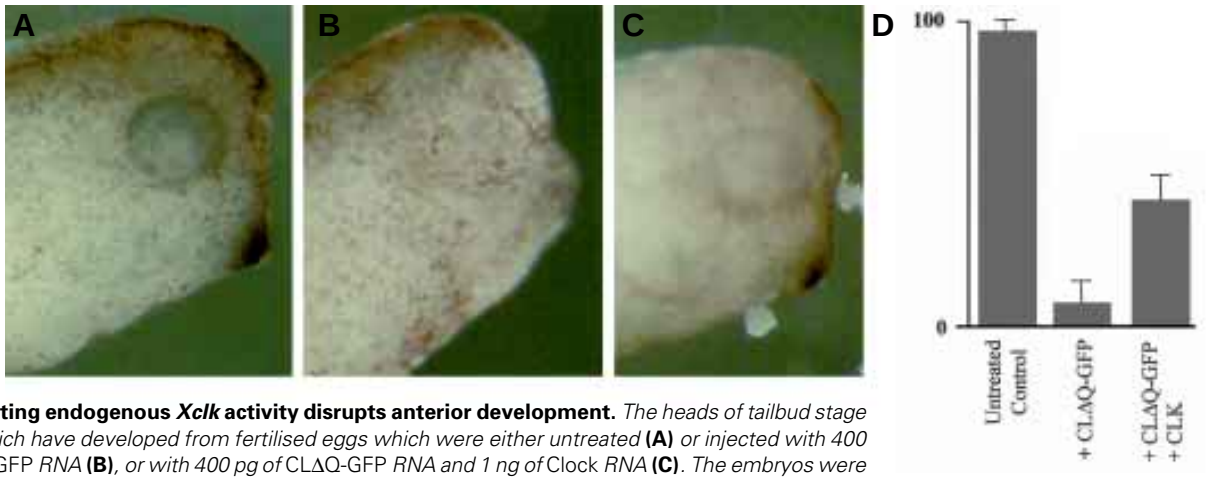

Fig. 2. Inhibiting endogenous *Xclk* activity disrupts anterior development. *The heads of tailbud stage embryos which have developed from fertilised eggs which were either untreated* **(A)** *or injected with 400 pg of* CLΔQ-GFP *RNA* **(B)**, *or with 400 pg of* CLΔQ-GFP *RNA and 1 ng of* Clock *RNA* **(C)**. *The embryos were allowed to develop to the tailbud stage. The proportion of embryos in each sample showing normal head morphology are shown in* **(D)**; *error bars represent the standard deviation from the mean of three separate experiments.*

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).

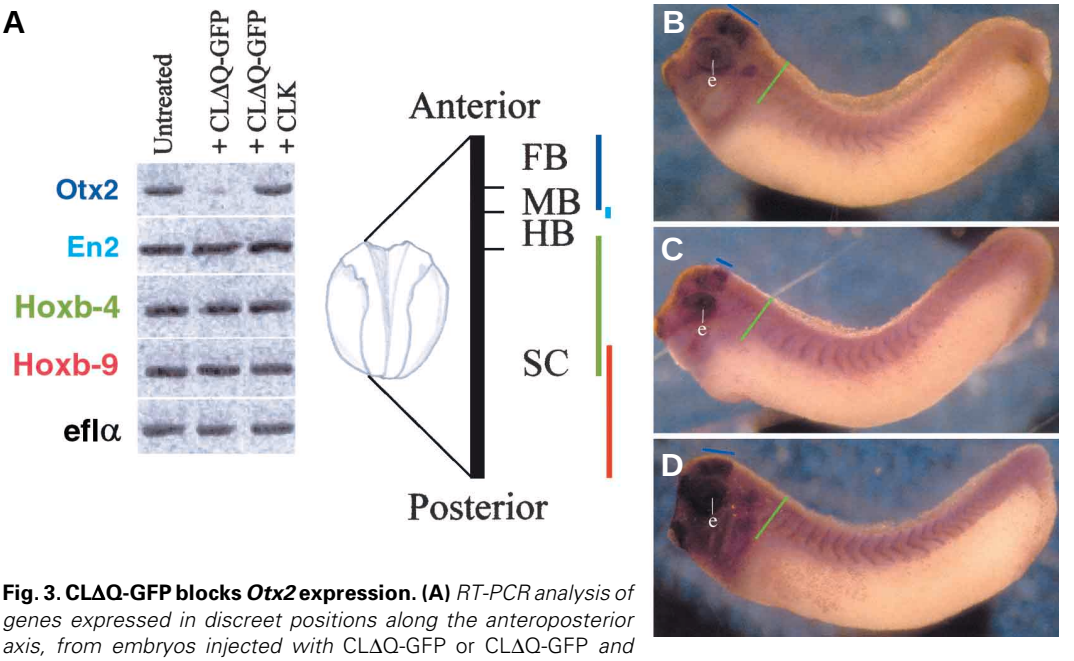

Fig. 3. CLΔQ-GFP blocks *Otx2* expression. (A) *RT-PCR analysis of genes expressed in discreet positions along the anteroposterior axis, from embryos injected with* CLΔQ-GFP or CLΔQ-GFP *and* Clock *RNAs as shown. The colour bars represent the expression domains of each of the genes shown. FB, forebrain; MB, midbrain; HB, hindbrain; SC, spinal chord.* ef1α *is included as a loading control. (B-D)* In situ *analysis of* Otx2 *and* Hoxb-4 *expression in the heads of tailbud stage embryos from eggs that were either uninjected* **(B)**, *injected with 400 pg* CLΔQ-GFP RNA **(C)**, *or injected with 1 ng* Xclk *RNA and 400 pg of* CLΔQ-GFP **(D)**. Otx2 *expression in the midbrain is indicated by the blue line, and the anterior limit of* Hoxb-4 *expression is marked by the green line. e, eye.*

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

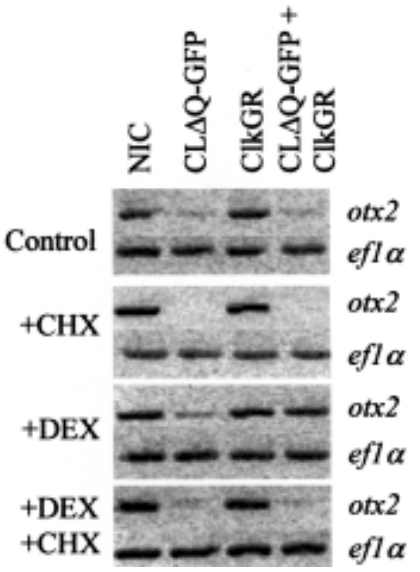

Fig. 4. (Left) RT-PCR analysis of *Otx2* and *ef1α* expression in neurula stage embryos. *Fertilised eggs were injected with either* CLΔQ-GFP *RNA,* CLK-GR *RNA, or both, and allowed to develop to the neurula stage. NIC, non-injected control. Before RNA extraction the embryos were treated with dexamethasone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

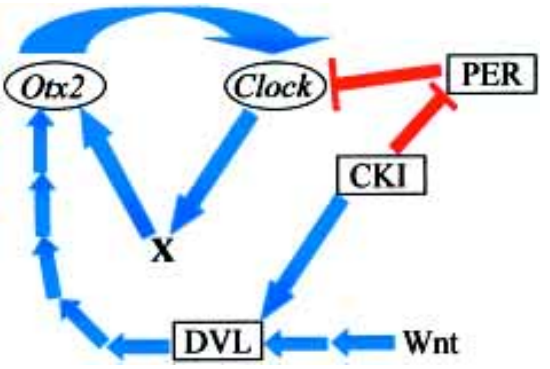

Fig. 5. (Right) Putative interactions between the circadian cycle and the wnt signal transduction pathway. *The blue arrows indicate positive interactions and the red lines indicate inhibitory interactions. The boxed components are proteins and those in ovals are genetic loci. PER, Period; CKI, Caesin Kinase I; DVL, Dishevelled.*

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 is similarly phosphorylated by the doubletime rotein kinase, which causes a reduction in the stability of PER. PER is rapidly degraded until the levels of the Timeless (TIM) protein accumulate and heterodimerize with PER, resulting in its stabilization. These heterodimers enter the nucleus where PER inhibits its own transcription by sequestering dCLK:dBMAL1 from the promoter (Lee *et al.,* 1998; Lee *et al.,* 1999; Bae *et al.,* 2000; Rothenfluh *et al.,* 2000). Since a PER:CRY complex forms before CRY enters the nucleus and inhibits transcriptional activation by CLK, the cytoplasmic retention of this complex by CKI may influence the increased transcription at CLK target genes, such as Otx2 (Fig. 5).

At the same time, a number of other groups found that CKI is a conserved component of the wnt signal transduction pathway (Peters *et al.,* 1999; McKay *et al.,* 2001; reviewed by Vielhaber and Virshup, 2001). The wnts are a family of secreted glycoproteins that have multiple roles in development and signal at least in part by a highly conserved signal transduction pathway (reviewed by Cadigan and Nusse, 1997). One of the positive components on this pathway is *dishevelled* (*dvl*). DVL protein becomes phosphorylated in response to wnt signaling and CKI may be the kinase that mediates this *in vivo* (Peters *et al.,* 1999; McKay *et al.,* 2001).

In light of these findings, it is noteworthy that *Otx2* expression is strongly upregulated by Wnt signaling (McGrew *et al.,* 1995). Furthermore, ectopic expression of *dvl* in early ectodermal cells strongly induces *Otx2* expression (Sokol *et al.,* 1995; Itoh and Sokol, 1997). It is tempting to speculate that the 'X' in Fig. 5 (i.e. the intermediate step in the activation of *Otx2* transcription by *Clock*) might also be a component of the wnt transduction pathway.

A recent study using Drosophila suggests that the segment polarity gene shaggy (the orthologue of vertebrate glycogen synthase kinase – 3 (GSK-3)) also has a role in the circadian clock (Martinek *et al.,* 2001). In Drosophila, PER protein is phosphorylated by the Doubletime 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 acts 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

Reverse Transcriptase (M-MLRvT) RnaseH- 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-MLRvT reaction (as described above), 0.2 nmols of each 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.

ClkGR *and* CLΔQ-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* / GR fusion (the two sequences being in frame with each other).

For CLΔQ-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 CLΔQ-GFP reading frame together with the SV40 poly A region from pEGFP-1was then amplified by PCR and the resulting product was cloned into pGEMT-easy (Promega) to give construct CLΔQ-GFPpGT.

Transcription of Capped mRNA for Microinjection

ClkGR RNA was transcribed from ClkGRCS2+ using SP6 polymerase after linearising with NotI, and CLΔQ-GFP RNA was transcribed using T7 RNA polymerase from CLΔQ-GFPpGT after linearising with BamHI. The transcription reaction was performed as previously described (Sive *et al.,* 2000). RNA was purified using the RNeasy kit (Qiagen).

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