Cryptochromes and Biological Clocks

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Many of the biological activities of living organisms are light dependent and revolve around the solar clock. A variety of behaviours and physiological processes are under the control of internal biological clocks. These internal clocks are remarkably synchronized with the external solar clock (day-night cycle of 24 hour periodicity). The molecular mechanisms of biological clocks have recently been uncovered. Although there are several molecules, which have a key role to play in the functioning of biological clocks, cryptochromes have been implicated to be of special importance. A detailed study of cryptochromes may provide some answers to problems related to the abnormal functioning of biological clocks in humans.

Circadian Rhythm

Many of our behaviors like sleeping or eating follow a set pattern every day and night and this pattern is repeated after fixed intervals of time. The same is true for many physiological processes like maintenance of body temperature and hormone production. Such repetition of biological phenomena in a periodic manner constitutes a *'biological rhythm'*. Many biological rhythms are synchronized with solar day (light hours) and night (dark hours). The day and night constitutes an external clock (environmental periodicity) of 24 hours, governed by the planetary movement of the Earth around the Sun, whereas the biological rhythm is an overt expression of an internal clock (*biological clock*). It is now recognized that the internal factors in the body set these rhythms and external signals like changes in light (day/night) synchronize them. It is interesting to know as to how the biological clocks respond to light stimuli and synchronize the daily activities to the environmental cycles.

Keywords
Cryptochrome, biological clock, circadian rhythm, chronobiology.

The oscillations in the behavioural, physiological and biochemi-

cal functions with a periodicity of approximately 24 hours have been termed 'circadian rhythms' (other rhythms may have periodicities greater or lesser than 24 hours). Circadian is derived from Latin, *circa* = around and *dies* = day. Circadian rhythms were first studied in 1729 by the Frenchman, Jean Jacques Ortous de Marian, who observed that the daily movements of a heliotrope plant persisted even when the plant was kept in the dark.

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Circadian rhythms are observed in most living organisms ranging from cyanobacteria to humans to plants, and this high degree of evolutionary conservation suggests that these rhythms or clocks confer some adaptive advantage to organisms possessing them.

The innate or free-running period of circardian rhythms (measured in a non-periodic environment eg. complete darkness), even though relatively constant for a population or species, varies among species and typically ranges from 22–25 hours.

Circadian rhythms have three basic features:

1. They are innate properties of the organism, are endogenously generated, and are maintained under constant (aperiodic) environmental conditions.

2. The period of circadian rhythms is temperature compensated, such that it is maintained at a constant value throughout the physiological range of external temperature.

3. Circadian rhythms can be synchronized or entrained to environmental light/dark cycle.

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Heat and other stimuli can also synchronize circadian rhythms with the external environment. However, it is the light/dark cycle, which is the most predominant external cycle and a major environmental stimulus for the synchronization of biological clocks. It is, therefore, important to know how the external stimulus of light is sensed by living organisms to synchronize the biological clock with the daily light/dark cycle.

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It is well known that many animals use light for vision. It is now certain that light is also being used to sense the time of the day to adjust the circadian rhythm. The information that is accumulating over 30 years, reveals that two photosensory systems exist in higher animals. The two systems, even though similar in receiving light stimuli, differ from one other with regards to the manner of processing the light stimulus in the brain.

Anatomy of Circadian Systems

The mammalian circadian system has been recognized to have three components (a) photoreceptor (b) oscillator (clock) and (c) output. Synchronization of the clock with the day-night cycle is so important that a number of organisms have multiple photosensory systems for circadian input pathway. Reptiles and birds have 3 or 4 photoreceptor organs namely, the eye, parietal eyes (reptiles), pineal gland and deep brain photoreceptors.

In mammals, all existing evidence indicates that photoreceptors for both vision and the circadian clock are located in the eye.

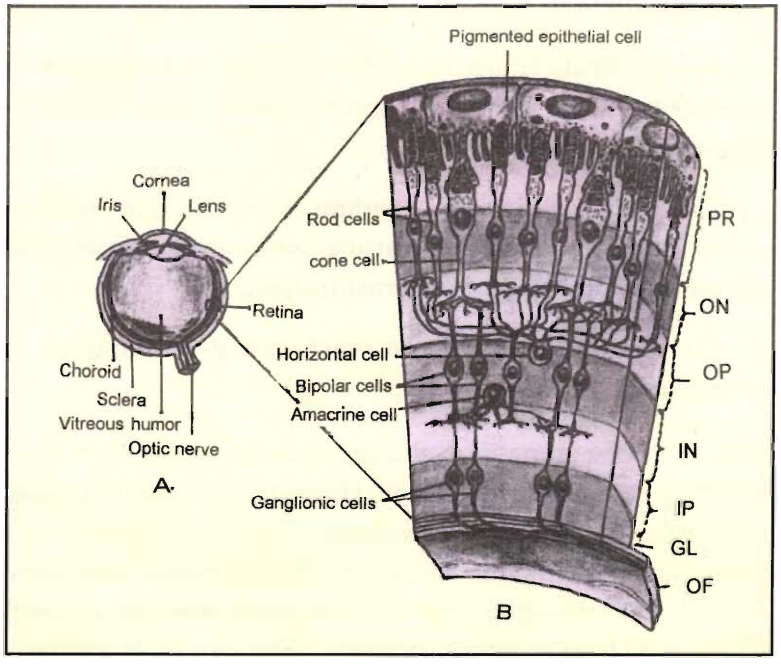

Figure 1. The anatomy of vision. A. Section through eye showing the location of light sensitive layer, the retina. B. The histological organization of cell layers in retina (PR – Photoreceptor layer; ON – Outer nuclear layer; OP – Outer plexiform layer; IN – Inner nuclear layer; IP – Inner plexiform layer; GL – Ganglionic layer; OF – Optic nerve fibres). The photoreceptors for vision are present in rod and cone cells in the outer pigmented epithelial layer, whereas the photoreceptors for the biological clock are located in the inner nuclear layer of the retina.

The photoreceptors for the two systems have different histological locations within the retina. The centres for processing the imaging and circadian photic input also have different anatomical locations within the brain.

Light for vision is absorbed by photoreceptor visual pigments (rhodopsin and colour opsins) in the rod and cone cells. These are located in the outer light sensitive pigmented epithelial layer. In contrast, light for synchronization of circadian rhythms is absorbed by special pigments in ganglion cells and cells in the inner layer of the retina, such as the amacrine cells, Muller cells and inter-neurons (*Figure* 1). The axons for vision continue their path in the optic nerve to the visual centers in the cortex, whereas the axons of the circadian system part from the optic nerve at the optic chiasm and go upwards to a pair of dense neuron clusters in the anterior ventral hypothalamus called the *supra chiasmatic nucleus* (SCN) (*Figure* 2).

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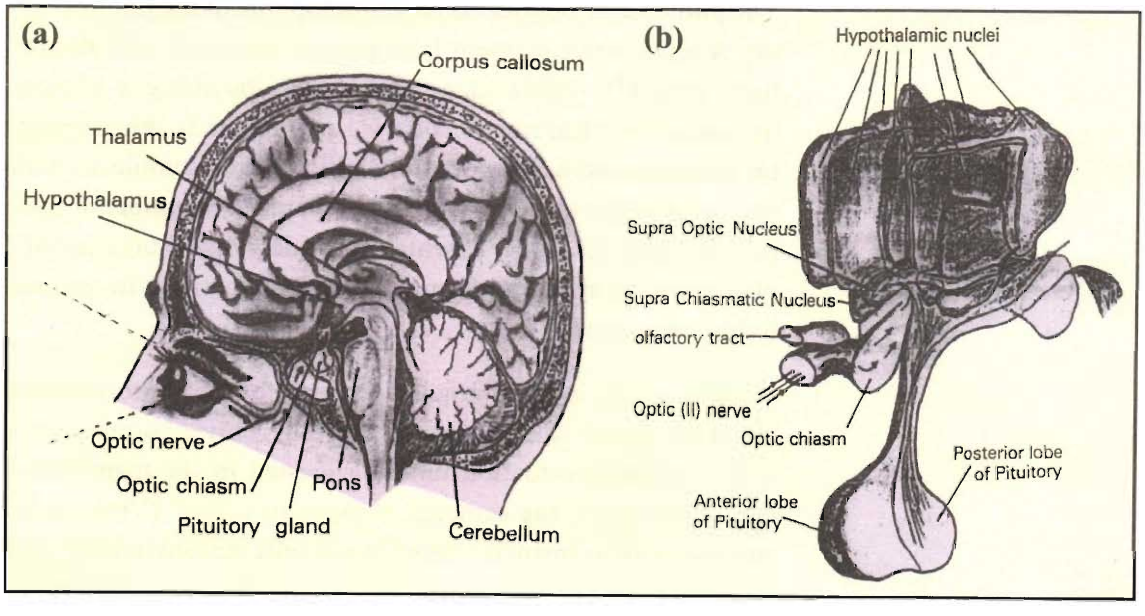
Figure 2. The anatomy of a biological clock in humans. The signals for visual perception travel via optic (II) nerve, enter brain through optic chiasm and reach visual cortex. While signals for circadian rhythm enter brain through optic chiasm and reach the supra chiasmatic nucleus (SCN), the master pacemaker organ, just above the optic chiasm, anterior to the supra optic nucleus, at the base of the hypothalamus.



Supra chiasmatic nucleus is now regarded as the master circadian pacemaker organ in mammals. The mechanism by which the oscillation in SCN is synchronized with the outside world is now believed to be through special photoreceptors called *cryptochromes*.

It has been shown that the circadian clocks are also located in other peripheral organs such as liver and lungs. However, these peripheral clocks do not work independently. The SCN overrides all local oscillations and coordinates the peripheral clocks with the central clock. A number of recent studies have demonstrated the unique pacemaker function of SCN in mammals, as opposed to other tissue clocks. It has also been demonstrated that SCN releases humoral factors that control peripheral oscillations. Thus, SCN is now regarded as the master circadian pacemaker organ in mammals. The mechanism by which the oscillation in SCN is synchronized with the outside world is now believed to be through special photoreceptors called *cryptochromes*.

Photoreceptors

The function of a photoreceptor is to capture light energy and transform it into a transportable form of energy. The conversion of light energy into chemical energy is brought about by various transduction mechanisms mediated by various photoreceptors. Basically, a photoreceptor is a complex of photoactive pigments and an apoprotein.

The photoactive pigment is the chromophore of the photoreceptor. A photoactive pigment is an organic molecule that absorbs light near UV-visible range and, upon absorbing a photon, initiates a chemical reaction (*Table* 1 and *Figure* 3). Photoreceptor proteins also have apoprotein which forms complexes with one or more photoactive pigments. There are a number of such receptor proteins (*Table* 2) which are widely distributed in both plants and animals, performing specific functions in various living organisms.

Until recently, it was thought that opsin based photoreceptors (rhodopsin and colour opsins) were the only photoreceptors with a phototransduction function located in the mammalian eye. Rhodopsin, the complex of pigment retinol (Vitamin A) and apoprotein Opsin, is located in rod cells that aid in black and

Name	Chemical nature	Biological significance	Distribution
1.Carotenoids	β-carotene (Provitamin-A)	Photoantenna pigment of photosysthesis.	Plants
		Catalytic pigments	Animals & bacteria
2. Retinol	Vitamin A	Visual perception	Animals
3. Bilins	Linear tetra pyrroles	Photoantenna in LHC	Plants
		Chromophore of phytochrome	
4. Chlorophylls	Cyclic tetra pyrroles	Photoantenna in LHC.	Plants
5. Flavins			
i. FAD	Coenzyme forms of Riboflavin	Photoactive cofactor of photolyase	Plants, animals and bacteria
ii.FMN		Chromophore of Phototropin	NPH gene product of *Arabidopsis*
iii.Deazaflavin	Similar to NAD	Photoantenna in photolyase	Cyanobacteria
6. Pterins	5,10-methenyl THF	Photoantenna in photolyase	Bacteria
7. Other photoactive pigments	*p*-OH Cinnamic acid	Photoactive pigment of phytochrome associated with GFP	Photosynthetic bacteria

LHC = Light harvesting complex of green plants. NPH = Non phototropic hypocotyl gene. GFP = Green fluorescent protein. THF = Tetra hydro folate.

Table 1. Photoactive pigments (chromophores).

white or dim light vision. Colour opsins, similar in structure to rhodopsin, are located in cone cells and aid colour or bright light vision. The circadian photoreceptor was assumed to be an opsin involved in vision as well in the circadian system, or a special opsin used specifically for the circadian system (*Table* 2).

Cryptochromes

Studies of the effect of light on organisms have revealed that blue light responses are remarkably universal in all species tested from bacteria to plants and animals. The various re-

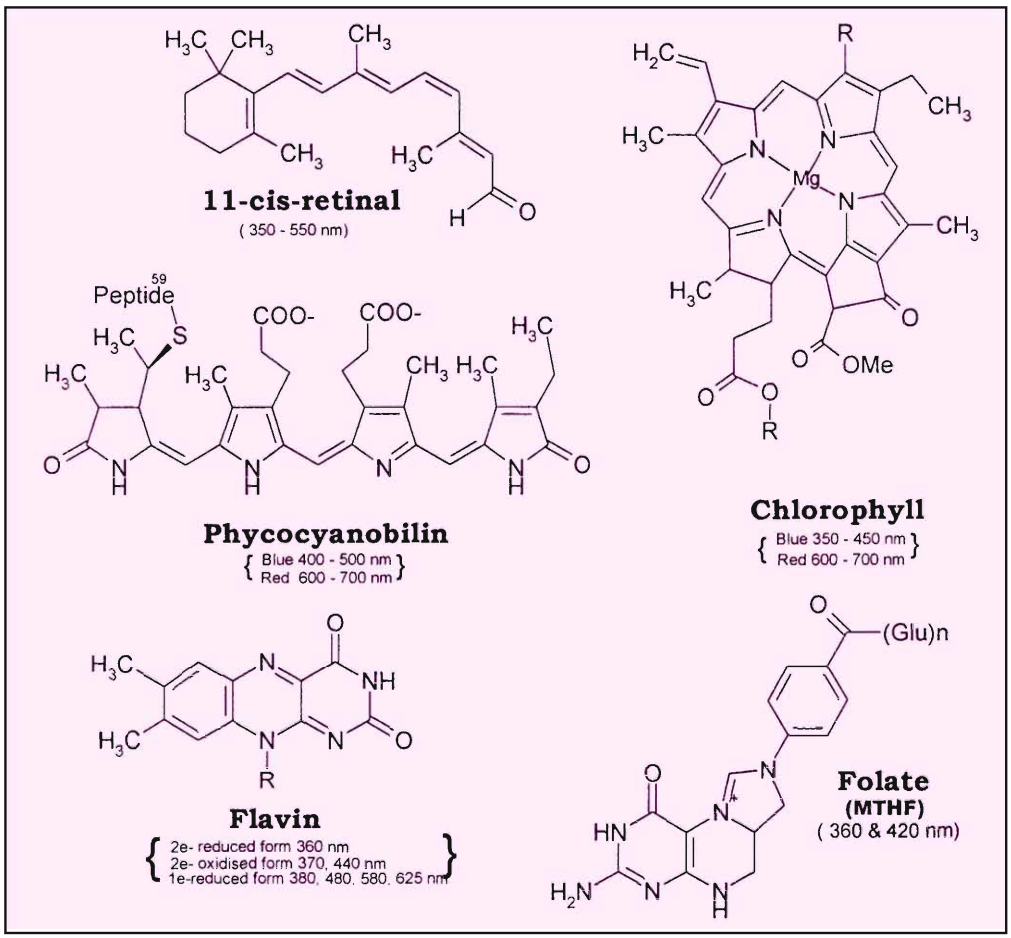


Figure 3. Structure of some photoactive pigments seen in living organisms.

sponses observed are photoactivation in bacteria, phototropism and photomorphogenesis in plants, phototaxis in protists, and circadian rhythms in fungi and *Drosophila*. Chronobiologists have searched for the circadian pigment using systems of varying complexities. The pigments system(s) responsible for many of the photoprocesses, especially for circadian rhythms, have been termed *cryptochromes*.

The first cryptochrome to be identified was photolyase, involved in DNA repair following UV induced damage (*Box* 1). In recent years, other cryptochromes have been cloned and characterized, such as HY4 and NHPI gene products of the dicot *Arabidopsis thaliana*. Both these proteins are flavoproteins and

Photoreceptor	Protein	Characteristics	Functions
1.Rhodopsin Cyanopsin Green opsin Erythropsin	Opsin	30-40 kD transmembrane protein, *cis-trans* isomerisation of retinol. Signal transduction through G protein.	Black & white and colour vision in higher animals.
2. Light harvesting complex (LHC)	Reaction centre polypeptide	Proteins associated with various photoantenna molecules – chlorophyll, bilins, carotenoids.	Photosynthesis in plants.
3. Phytochrome	Protein with kinase activity	125 kD homodimer linked with bilins, with Ser/Thr or His at the active site.	Photomorphogenesis in plants and cyanobacteria.
4. Phototropin	Protein with kinase activity	120 kD membrane protein containing FMN.	Regulation of phototropism in response to blue light in plants.
5. Photolyase	Protein with enzyme activity	55-65 kD monomeric, cytosolic protein containing flavins (FADH) and pterins (Methenyl THF).	Repair of DNA damage caused by UV light (350-450 nm).
6. Cryptochrome	Simple protein.	55-65 kD monomeric protein identical to photolyase but without any enzymatic activity. Contains flavins and pterins.	Regulation of circadian rhythm in living organisms.

Table 2. Photoreceptor proteins.

the latter is called *phototropin*. Thus, at present there are three blue light photoreceptors that have been genetically, and to varying degree, biochemically characterized: photolyase, HY4 protein and phototropin. All these molecules qualify to be called cryptochromes. The first cryptochrome gene was isolated from mustard (*Sinapsis alba*) as a sequence homologue of photolyase and was thought to be the gene for the mustard photolyase.

Box 1. Photolyase: Repair of radiation damage of DNA

DNA strands, when exposed to radiation in the near UV to blue light range (350-450 nm), sustain damage. This damage occurs when pyrimidine bases in the nearby strands form dimers. Two types of dimers are formed. One type is cyclobutane dipyrimidine dimer, while other type is pyrimidine-pyrimidone 6-4 photoproduct. These dimers become a block during gene expression (transcription). Living organisms have evolved various repair systems for such damage, comprising specific enzymes. One such enzyme is *Photolyase*. There are two types of photolyases. One type repairs cyclobutane dipyrimidines, and is found in many bacteria, some eukaryotes and a grasshopper virus, whereas the other type repairs the 6-4 photodimer and is found in *Drosophila*, *Xenophus*, rattle snake, fish and *Arabidopsis*. Both types of photolyases contain two photoactive pigments. One is invariably FADH while the other one is a pterin (methyl tetrahydrofolate).

The pyrimidine dimers are recognized by the photolyase in a light independent manner. The enzyme then forms Michaelis complex with the substrate. On exposure of the complex to light, the second chromophore of the photolyase absorbs and transfers the excitation energy to flavin, which in turn, transfers an electron to the DNA photoproduct: the cyclobutane ring of pyrimidines. Back electron transfer restores FADH neutral radical to the catalytically competent FADH form. The enzyme then dissociates from the DNA strand to enter new cycle of catalysis.

Two human genes (*hCry1* and *hCry2*) have recently been identified that encode proteins which have similarity in amino acid sequence with photolyase. The proteins (hCRY1 and hCRY2) have similar chromophores (FAD and pterin) but they do not have enzyme activity. The proteins are presumed to mediate circadian clock in humans and are also *cryptochromes*. Thus, cryptochrome has now assumed a precise definition: "A photoreceptor with sequence homology to photolyase but with no enzymatic activity and that mediates other blue light responses."

In contrast with plants, in which essentially all aspects of development and behavior have light regulated, photoregulated physiological responses in humans are limited to vision and photoentrainment of the circadian clock. Hence, it was proposed that hCRY1 and hCRY2 may be the circadian photoreceptors of humans.

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Structure of Cryptochrome

The two human cryptochromes discovered have now been partially characterized. The hCRY1 is a 586 amino acid long 66 kD

protein, whereas hCRY2 is a 593 amino acid long 67 kD protein. Cryptochromes from various sources have remarkable high sequence identity. Human, *Drosophila* and *Arabidopsis* cryptochromes have about 60% sequence identity. Apart from this, cryptochromes from different sources have non-homologous c-terminal extensions ranging from very short ones in *Drosophila* to as many as 240 amino acids in plants. The two human cryptochromes are 73% homologous to each other. However, they exhibit no sequence homology among the c-terminal 75 amino acids. It is thought that this domain may bind to effector molecules.

The crystal structure of human cryptochrome has not been determined in detail. However, the crystal structure of *E.coli* photolyase is determined to reasonable details (*Figure* 4), and is superimposable to human cryptochrome. Hence it is reasonable to assume that hCRY structure would be similar to *E.coli* photolyase. The *E.coli* enzyme has an overall dimension of 80 × 60 × 30 Å and consists of two well defined domains interconnected by a loop of 62 amino acids. The *N*-terminal α-β domain adopts a fold (Rossman fold). The pterin cofactor is located in the inter-domain cleft near the surface and makes intimate contact with residues in the α-β domain. The C-terminal helical domain is made almost entirely of α-helices and resembles a slab of 60 × 40 × 20 Å. The prominent feature of the α-β domain is a hole in the centre of the flat surface that leads to FAD in the bottom of the hole. The FAD has a 'U' shaped or *cis* conformation with the isoalloxazine and adenine rings in close proximity. This is important and unique to proteins of the photolyase/blue light photoreceptor family which carry out catalysis from an excited state.

The molecular modeling of hCRY1 and hCRY2 on to the α-backbone of *E. coli* photolyase indicates that the overall geometry, including the hole in the centre, is retained in these proteins.

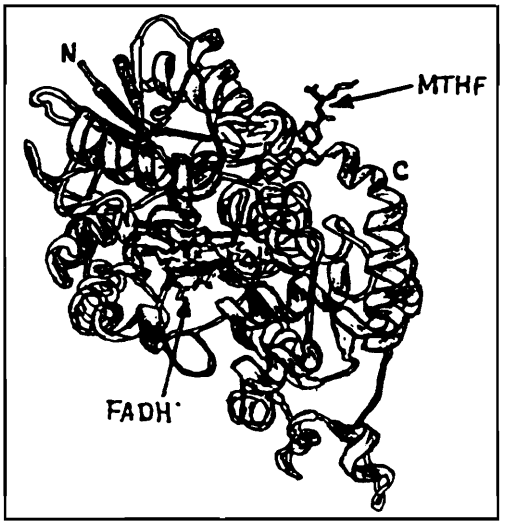

Figure 4. The 3D structure of E. coli photolyase. The photoantenna Methenyl tetrahydrofolate (MTHF) is located in the crevice between the α-β domain and the α-helical domain and is exposed to solvent. The $FADH^-$ catalytic factor is in cis conformation (adenine ring stacked on flavin ring) and is buried deeply within the α-helical domain. The centre-to-centre distance between the two chromophores is 17Å. The structure of human cryptochrome is almost identical to the E. coli photolyase.

Many components of the circadian clock have been identified and characterized. At present eight human and mouse genes that qualify for the definition of clock genes have been identified, isolated and characterised

These observations raise an interesting possibility that cryptochrome has retained the unique feature of the photolyase reaction mechanism i.e. dinucleotide flipping and photoinduced electron transfer.

Location of Cryptochromes

All known animal clock proteins are located either in the nucleus or shuttle between cytosol and nucleus to exert their feedback in to the circadian (transcription) loop. Thus, the location of a potential photoreceptor molecule is important from the standpoint of phototransduction mechanism. From the available studies, it appears that cryptochromes are located in the nucleus. The nuclear photoreceptor can interact directly with the clock genes and proteins and thus function both as photoreceptor and photoinducer. CRY1 and CRY2 are essential components of the mammalian clock which reside in nucleus and appear to function by the above mechanism.

Molecular Model of Mammalian Clock

Many components of the circadian clock have been identified and characterized. At present eight human and mouse genes that qualify for the definition of clock genes have been identified, isolated and characterised (*Clock, Bmal1, Per1, Per2, Per3, Tim, Cry1* and *Cry2*). From the initial characterization of these genes and their proteins, a reasonably detailed molecular model for the mammalian circadian clock has been developed (*Figure* 5).

At the cellular level circadian rhythms arise through an autonomous and auto-regulatory transcription loop. The main feature that distinguishes the circadian negative feedback loop from the biochemical feedback loop is that the circadian system has a time delay between the transcription of the clock genes and the production/availability of negative feedback inhibitory protein. This is accomplished either by delay in translation, a delay in activation by post translation modification, or delay in the entry of the feedback inhibitor in the nucleus.

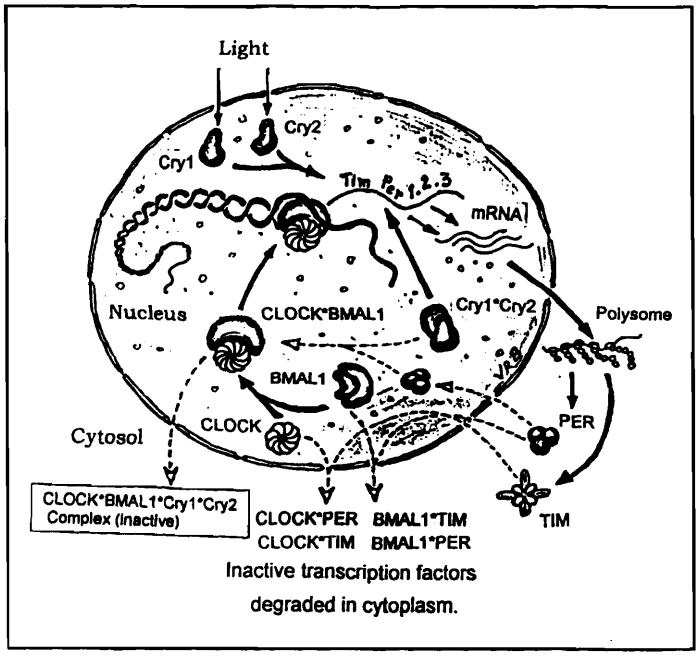

Figure 5. Molecular model of the mammalian circadian clock. The control mechanism of the clock is located in the nucleus. Cryptochromes mediate the light induction of Per genes by direct interaction in the nucleus (Positive drive). In addition, they may dimerise with PER and directly interact with CLOCK × BMAL1 complex and function as negative feedback loop as well. The balance between positive regulatory effects (_____) and negative feedback loops (.....) account for the operation of the clock.

Tim and *Per* are the clock genes, which encode TIM, PER1, PER2 and PER3 proteins. CLOCK and BMAL1 are the positive transcriptional protein factors, which form a complex. This dimer complex, CLOCK × BMAL1, binds to a specific region of the *Tim* and *Per* genes. This activates and switches on transcription so that clock genes are expressed. However, there is delay in the translation of these genes in cytosol by about 6 hours from the initiation of maximum transcription.

When the products of *Tim* and *Per* genes are formed in cytosol, they enter the nucleus and form heterodimers such as PER × TIM or PER × CRY. These dimers interfere with the activator function of CLOCK × BMAL1 by prevention of CLOCK × BMAL1 complex formation. This is achieved by formation of heterodimers such as CLOCK × PER, CLOCK × TIM, BMAL1 × PER and BMAL1 × TIM. These heterodimers are inactive transcriptional factors and are degraded in cytosol. As a result the *Tim* and *Per* genes are switched off and the corresponding proteins (TIM and PER) are degraded. When the levels of TIM and PER fall, and levels of CLOCK × BMAL1 rise, a new cycle starts.

The molecular level, cryptochromes play a dual role in the operation of biological clocks. They function as photosensitive input molecules, as well as negative feedback regulators of the clock genes.

CRY1 and CRY2 proteins play a central role in the cycle. Cryptochromes mediate light induction of *Per* genes and may positively affect the steady state cycling of *Per* genes. In addition, independent of their photoreceptor function, they dimerise with PER and perhaps directly interact with CLOCK × BMAL1, thus functioning in the negative feedback loop as well.

Finally, differences in the relative contributions of CRY1 and CRY2 in the positive drive and negative feedback component of the loop must account for the differences in their influence on circadian clock. Thus, cryptochromes are the photoreceptors and essential components of the circadian oscillator, performing partly redundant and partly complementary function. Currently it is not known how the cryptochromes transmit light signal to the molecular clock

Concluding Remarks

With the available information on clock genes, a reasonable model for the circadian clock and the system has been proposed that appears to be based on auto-regulatory transcriptional-translational loop. It is now clear that, at the molecular level, cryptochromes play a dual role in the operation of biological clocks. They function as photosensitive input molecules, as well as negative feedback regulators of the clock genes. The study of clock setting by cryptochromes may reveal a novel signal transduction mechanism whose significance could transcend the field of circadian research.

Suggested Reading

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