

## Identification of circadian gene expression in the rat pineal gland and retina by mRNA differential display

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Received 23 October 1994; revised version received 15 December 1994; accepted 6 January 1995

### Abstract

To identify unique gene products that could be crucial for circadian and light-dark regulations, using the technology of differential display, we have investigated mRNAs that were isolated from rat pineals and retinas collected either during subjective day (D) or subjective night (N). A subpopulation of about 50 mRNAs were amplified from pineal using designed primers and reverse transcriptase-polymerase chain reaction. Thirty five of the mRNAs were expressed equivalently in both D and N samples. From the 15 mRNAs showing differential expression, four amplified products were selected, based on higher expression during the subjective night (N310, N320, N383, N420) and each was subcloned and sequenced. Of the four cDNAs studied, only N310 was determined to have significant identity to a known cell adhesion protein, called F3. This protein is highly expressed in developing mouse neurons and is related to chicken contactin. Each differentially displayed product identified is being examined further in order to understand their potential significance in the circadian regulation of pineal and retinal activities.

**Keywords:** Pineal gland; Retina; mRNA differential display; Protein F3; Circadian rhythm

Sensitivity to ambient lighting conditions links the functional activity of many organisms to day/night fluctuations in solar energy. In lower vertebrates, both the retina and pineal gland provide light sensitivity to the organism, whereas light sensitivity in mammals is restricted to the retina. Opacity of the mammalian skull necessitates that neuronal information pertaining to environmental lighting reaches the pineal gland through a multisynaptic neuronal network that is synchronized by a biological clock [22] within the suprachiasmatic nucleus [4,13] of the hypothalamus and is modifiable by light during the hours of darkness. A neuronal signal is then transduced within the pineal into a dramatic nocturnal increase in the metabolism and secretion of melatonin [7,23,25]. Ample documentation exists suggesting that melatonin plays a major role in the regulation of both circadian and seasonal rhythms [12,20,26]. Despite their anatomical and functional divergence in mammals, photo-

receptors of the retina and pinealocytes of the pineal show signs of a common embryonic ancestry. Both remain essential in the regulation of biological rhythms, utilizing some shared mechanisms of signal transduction and an intrinsic capability for the synthesis and secretion of melatonin [7,17,19,21,28]. The local and intrinsic signals that coordinate circadian rhythms within the pineal and retina may have a common origin also. To analyze these similarities, we have used a differential display technique. This new technology is based on the assumption that every single expressed mRNA species can be reverse-transcribed and PCR-amplified [16,27].

The general strategy of differential display is to use a set of arbitrary primers to amplify only a subgroup of cDNA sequences from the total mRNA pool. For the reverse transcriptase step, a poly-T11 flanked by two additional 3' bases is used as a 3' primer (dT11XX). By probability, this primer will transcribe one-twelfth of the poly A<sup>+</sup>-mRNA population [14–16]. The synthesized cDNAs will then be PCR amplified by using the original set of dT11XX and a second, arbitrary (10–12 bases) primer to provide annealing positions that are randomly distributed 5' of the poly-A tail [16]. The final PCR products, labeled

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with [ $\alpha$ - $^{35}$ S]dATP, are visualized by autoradiography after electrophoresis through a denaturing polyacrylamide gel. The cDNA fragments preferentially expressed are eluted from the gel and reamplified for further analysis. The present results describe, in both the rat pineal gland and retina, some newly identified mRNAs differentially expressed on a circadian basis.

Sprague–Dawley male rats (Zivic-Miller) were reared on a 12 h/12 h light/dark cycle and kept in constant darkness the day before the experiment. They were killed by means of hyperbaric carbon dioxide asphyxiation under a dim red light at circadian time (CT) 3 and CT 15. Tissues were rapidly dissected and frozen on dry ice. Total RNA was extracted by using RNAzol (Tel-Test, Inc.) according to the protocol of Chomczynski and Sacchi [6]. One microgram of total RNA was reverse transcribed in a reaction volume of 10  $\mu$ l, containing 2  $\mu$ M dT11GC, 20  $\mu$ M of each dNTP (Perkin Elmer Cetus), 200 units of M-MLV reverse transcriptase (Gibco-BRL), 50 mM Tris–HCl (pH 7.5), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM dithiothreitol (USB), 10 units of RNasin ribonuclease inhibitor (Promega). The samples were incubated at 37°C for 60 min and terminated with a 5-min incubation at 95°C.

The two sets of cDNAs were amplified in a total volume of 25  $\mu$ l. The PCR mixtures contained: 2.5  $\mu$ M dT11GC 3'-primer, 0.5  $\mu$ M 5' arbitrary primer (5'-TGT-TCCTTGCA-3'), 1  $\mu$ l of cDNA, 80  $\mu$ M of each dNTP, 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl<sub>2</sub>, 5  $\mu$ Ci [ $\alpha$ - $^{35}$ S]dATP (Dupont NEN) and 0.625 units of Taq DNA Polymerase (Promega). The PCR conditions were: 40 cycles at 94°C for 1 min, 42°C for 1 min and 72°C for 1 min, followed by an extension period at 72°C for 1 min.

Two microliters of each sample were mixed with 3  $\mu$ l of 95% formamide/dye solution and heated at 95°C for 5 min. Three microliters of this mixture were electrophoresed on a standard sequencing gel (6% polyacrylamide, 7 M urea) during 4–5 h in a 1 $\times$  TBE buffer (100 mM Tris, 90 mM boric acid, 2 mM EDTA) at 1800 V. A 1-kb DNA ladder (Gibco-BRL) labeled with terminal transferase and [ $\alpha$ - $^{32}$ P]dCTP was used to determine the size of PCR products. Gels were washed for 20 min in a 10% acetic acid, 10% methanol solution, followed by a 10- and 5-min wash in water. Gels were dried directly on a previously silanized glass plate and exposed to X-ray film for 1–5 days.

The bands selected from the autoradiograms were cut from the dried gel and rehydrated with 20  $\mu$ l of TE buffer. One half of the sample volume was reamplified with the identical PCR conditions and primers. The size and purity of the fragment were analyzed by 0.8% agarose gel electrophoreses in 1 $\times$  TBE buffer. The amplified product was visualized with ethidium bromide staining.

These reamplified PCR products were subcloned into a pCR<sup>TM</sup>II vector (TA cloning Kit, Invitrogen). Both strands of the cDNA fragment were sequenced (Seque-

nase, version 2.0 DNA sequencing kit, USB) using SP6 and T7 promoter primers, according to the protocol of the manufacturers.

For the RNA analysis, 5  $\mu$ g of total RNA was electrophoresed on a 1.5% formaldehyde-agarose gel with 1 $\times$  MOPS buffer. The gels were electroblotted overnight onto a Zeta probe membrane (BioRad). The filters were baked in an 80°C vacuum oven for 15 min. The blots were prehybridized, hybridized and washed as previously described [8].

After polyacrylamide gel electrophoresis of the RT-PCR products, between 40 and 60 bands were displayed on the autoradiograms (Fig. 1). The reproducibility of the technique in displaying similar patterns was verified by using two different sources of mRNA samples. The results indicated that approximately 90% of the amplified fragments were shared between subjective night and subjective day mRNA populations (Fig. 1). The majority of

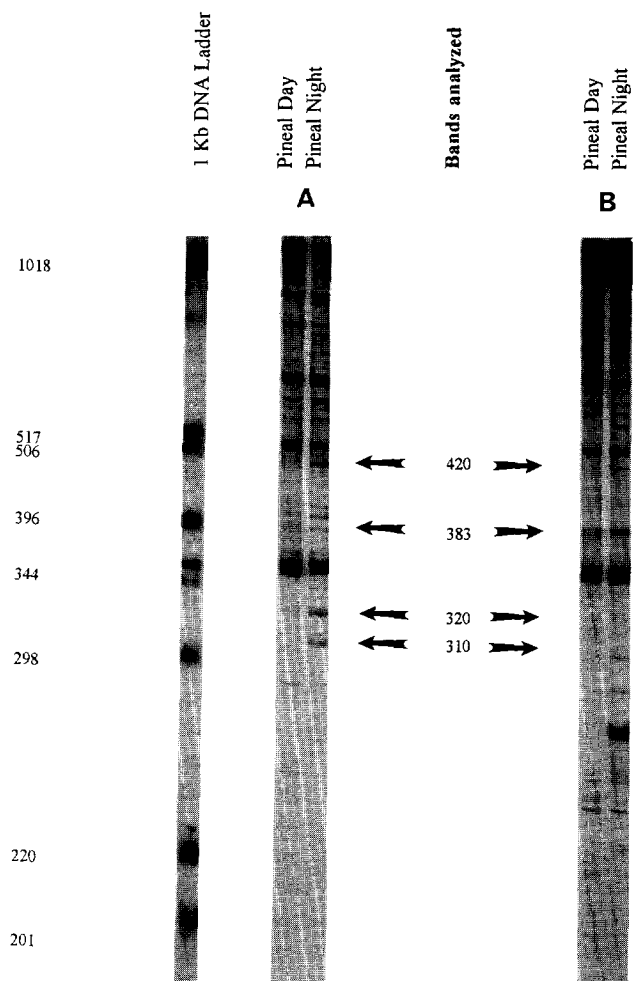


Fig. 1. The observed band pattern was derived from cDNA synthesized from total RNA extracted from either subjective day or subjective night rat pineal using the differential display RT-PCR technique. The displays (A) and (B) were obtained with two different preparations of mRNA (10 pineals each) to verify the intra-experimental reproducibility of the technique.

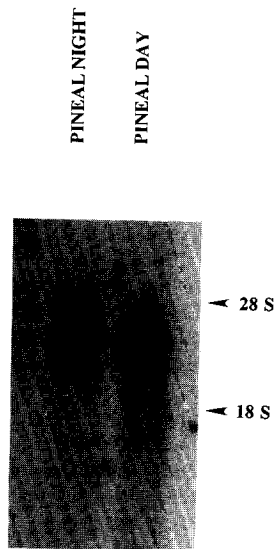


Fig. 2. Northern blot hybridization of day and night adult pineal with total RNA was probed with a radiolabeled N310 cDNA probe. The open arrows indicate the specific hybridization signal of the mRNAs. The mobility of the 18 S and 28 S ribosomal bands are shown on the right.

the bands, around 70%, were of similar intensity in both samples. The remaining bands were either exclusively or more highly expressed during subjective night time compared to subjective day time, or vice versa. Four of the most prominent fragments, differentially expressed during subjective night, were selected and analyzed: N310, N320, N383, N420 (Fig. 1). The nucleotide sequence analysis of both N320 and N383 revealed A/T rich sequences, suggesting 3' non-coding sequence, and Northern blot analysis were below detectable levels. The database search revealed no sequence homology with other known mRNAs. On the contrary, the sequence from the cDNA of N310 shared high identity with a known protein. The nucleotide sequence has, respectively, 91% and 82% identity with the 3'-coding region and the beginning of the non-coding region of the mouse mRNA transcript of F3, a neuronal cell surface protein [10]. The translation of the amino acid sequence reveals 94% identity. Northern blot hybridization of pineal RNA with a probe of N310 revealed a band specifically expressed during subjective night in the 6 kilobase (kb) region, plus a shared mRNA species in both subjective day and night in the 3 kb region (Fig. 2). Similar RNA species in mouse brain have been reported by others [10]. These results strengthen the fact that the pineal isolated band is a part of the mRNA of F3 and is expressed in the pineal. Previously, F3 was shown to be a phosphatidylinositol-anchored member of the immunoglobulin superfamily and related to the chicken contactin [10]. The F3 protein is known as a neuronal cell adhesion molecule [11], most closely resembling L1. Its soluble form promotes neurite outgrowth on an *in vitro* model of mouse dorsal root ganglion neu-

rons [9]. Increased night time expression of F3 in pineal glands of the adult rat appears to be higher than in other brain areas reported, as suggested by both our northern analysis and differential display. These results are in contrast to data described in the mouse brain, where F3 expression peaks during the second postnatal week of life, followed by a precipitous eightfold drop in adults [10]. F3 has been proposed to play a major role in cell surface interaction during nervous system development [10]. Whether or not F3 interacts with the development of the adrenergic innervation of the pineal gland will be investigated. The functional aspect of its circadian fluctuations in the pineal gland remains enigmatic and will require additional experiments.

The nucleotide sequence of clone N420 has no sequence identity in the database (NCBI Blast Network, 9/27/94) [1]. PCR amplification of cDNA synthesized with oligo-dT as a primer revealed circadian fluctuations in both pineal gland and retina with a higher expression during subjective night time (Fig. 3). A weak signal was also observed in the liver during the subjective night time. No other tissue had detectable levels of mRNA expression during subjective day or night time (Fig. 3). Since, in both pineal and retina, these day/night fluctuations were observed in animals kept in constant darkness for 24 h prior to the sacrifice, these variations possess fundamental characteristics of a circadian rhythm [2,5,24] and are driven by the suprachiasmatic nuclei circadian activity [18,24].

We report here the use of the differential display RT-PCR to identify differentially expressed mRNAs from two time points during the 24-h cycle in rat pineal gland. Four mRNA species, specifically overexpressed during

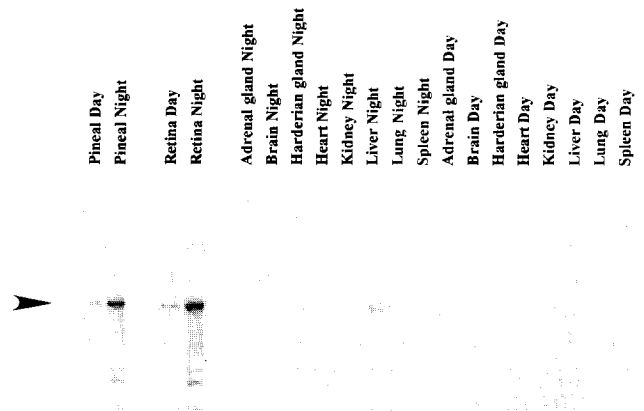


Fig. 3. PCR band pattern derived from cDNA transcribed from total RNA isolated from subjective day or night pineals, retinas and other body tissues. The cDNAs were synthesized using oligo dT as a primer (2.5  $\mu$ M). The cDNA was diluted 1/10 before the PCR amplification. The two primers (5'-N420-21mer, and 3'-N420-21mer) used for amplifying the cDNAs were designed from the 5' and 3' extremity of the nucleotide sequence of the band N420. The primers and dNTP concentration were 1  $\mu$ M and 80  $\mu$ M, respectively. PCR parameters were: 30 cycles of 94°C, 1 min; 52°C, 1 min; 72°C, 1 min followed by 10 min at 72°C. The samples were electrophoresed as described in the text.

subjective night, were studied. The differential display offers unique advantages over subtraction-based screening methodology [27]. First, since this method is PCR based, the results can be obtained with small quantities of total RNA [3,14–16,27]. This advantage is crucial, especially when working with limited sample size such as the pineal gland, which contains minute quantities of RNA. Secondly, although two of the clones identified in this study, N310 and N420, are dramatically overexpressed during night time, they are also present during day time. In this particular case, the use of subtractive hybridization between subjective day and night isolated mRNA would not have identified these mRNAs [27]. Indeed, in a subtractive hybridization, since the target tissue cDNA is hybridized in solution to an excess amount of complementary RNA or cDNA derived from the driver tissue [27], one can postulate that signal intensity of both N310 and N420 would have been dramatically reduced to an undetectable level.

An unclarified aspect of the differential display method is sensitivity. According to the theory, every single expressed gene should be present in the patterns with the use of the correct set of primers [16]. Strengthening the mathematical theory, some results suggest that, by using a given set of primers, one can obtain a higher sensitivity with this new method compared to the method using a cDNA library construct [16]. However, a particular cDNA has to contain a complementary sequence for the 5'-primer to be amplified. Two other limitations should be considered: (1) the 3' primer used anchors to the 3' extremity of the mRNA (the poly A tail), the probability of getting mostly non-coding sequences in the expressed bands is high. In some cases, the complete band isolated and subcloned can be non-coding, as is the case for N320 and N383. (2) Since this technique is PCR-based, it is only semi-quantitative and sometimes it yields false positive products [16]. This latter point was discussed by Liang et al. [15].

The mRNA differential display technique is an excellent methodology to provide a selection of new candidate genes to investigate the genetic control of circadian rhythmicity. The newly identified mRNAs described in this study are being examined further to understand their potential functional role in the light/dark regulation of pineal and visual signal transduction, as well as in the regulation of circadian rhythms.

Research support provided by grants from Ella C. McFadden charitable trust and NIH R29 NS28126. This work is dedicated to Mary D. Allen for her generous support of vision research. We thank To Hoa Thai for his excellent technical assistance and Dr. Richard N. Lolley for editorial comments.

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