

cDNA Array Analysis of Pineal Gene Expression Reveals Circadian Rhythmicity of the Dominant Negative Helix-Loop-Helix Protein-Encoding Gene, Id-1

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Abstract

The pineal gland is a major output of the endogenous vertebrate circadian clock, with melatonin serving as the output signal. In many species, elevated nocturnal melatonin production is associated with changes in pineal gene expression. In the current study, cDNA array analysis was used in an attempt to identify additional genes that exhibit day/night differential expression in the rat pineal gland. This revealed 38 candidate genes, including Id-1 (inhibitor of DNA binding and differentiation). Id-1 encodes a helix-loop-helix (HLH) protein that lacks a basic DNA binding domain and could affect pineal physiology via a dominant negative *trans*-acting regulatory activity. For this reason Id-1 was selected for further analysis. Id-1 was expressed in a major population of pineal cells and the Id-1 protein was associated with a nuclear complex. The levels of Id-1 mRNA and protein exhibit approximately six-fold day/night rhythms. In contrast, the related genes Id-2 and Id-3 do not exhibit marked day/night differences in pineal expression. Rhythmic Id-1 expression is primarily limited to a C-terminally extended splice variant of Id-1, which would restrict the functional output of the rhythm to protein binding partners of this isoform of Id-1. Our findings add to the body of evidence indicating that transcriptional regulators play a role in neuroendocrine rhythms, and extend this by introducing the concept of a dominant negative HLH involvement. The rhythm in Id-1 in the pineal gland provides an experimental opportunity to identify Id-1-binding partners which may also be involved in Id-1 activity in other functional contexts.

Biological systems are functionally sustained through precise temporal organization. Circadian (approximately 24 h) rhythms are now recognized as a common element of such organization in most forms of life. Circadian biology is currently being advanced through the identification of gene products which constitute the three main components of circadian clocks, those involved in detecting photic input, generating pacemaker activity, and in producing output (1).

In the mammalian circadian system, the retina detects photic input. Circadian pacemaker activity is generated within the suprachiasmatic nucleus (SCN) – the mind's clock; signals from the retina are sent to the SCN via a retinohypothalamic projection. A neural circuit comprised of central and

peripheral structures links the SCN with the source of the major output of the vertebrate circadian clock, melatonin (1). Melatonin synthesis and release follows a circadian pattern, with high levels occurring at night. The rhythm in melatonin production is regulated by the serotonin-acetyating enzyme, arylalkylamine *N*-acetyltransferase (AA-NAT), which is the focal point of regulatory mechanisms (2–4), and serves as a molecular transducer.

In addition to AA-NAT, other aspects of pineal biochemistry exhibit circadian rhythms, and it is suspected that all contribute to the precise control of the circadian rhythm in the production of melatonin. An interesting example of this comes from the rodent pineal gland, where circadian changes occur

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in transcriptional regulatory mechanisms involving phosphorylated CREB and the CREM isoform, ICER (5–7), AP-1 family proteins (8–10) and homeobox proteins (11); some of these changes are involved in the transcriptional regulation of AA-NAT mRNA levels. Regulation also occurs at a post translational level through control of proteasomal proteolysis of AA-NAT (12), which occurs in response to light exposure at night, causing the rapid degradation of AA-NAT. This is essential for the circadian pattern in melatonin production, and because of this, proteins involved in AA-NAT proteolysis may be under precise circadian regulation.

Elucidation of the molecular mechanisms underlying rhythmic pineal function has been advanced by the application of differential selection strategies (13–15). The large daily rhythmic pattern in activity and gene expression facilitates such approaches, as does the homogeneous cellular population of the pineal gland. In this study, gene array technology was used, which has the advantage over the aforementioned techniques in providing simultaneous expression profiles of both characterized and novel transcripts at a partial transcriptome-level of resolution. As described, this approach identified 38 genes that are differentially expressed on night/day basis. One of these, Id-1, was studied in detail. Id-1 is a helix-loop-helix (HLH) protein known to interact with basic (b) HLH proteins and to prevent DNA binding and consequent transcriptional regulatory activity through E-box *cis*-acting elements; this is of special interest because E-box elements are known to mediate circadian control of gene expression (1).

Methods

Animal protocols

Animal studies were conducted in accordance with both UK Home Office regulations, and local ethical review. Adult (aged 3–4 months), male Sprague-Dawley (CD) rats were maintained in standard laboratory conditions either in a 14:10 h light:dark cycle (lights on: 05.00 h) or, for certain experiments, in constant darkness for a period of 3 days. Animals were killed by cervical dislocation at appropriate times of the daily cycle, and tissues were rapidly dissected. Sample time points for the initial array analysis (light 12.00 h and dark 24.00 h) were selected according to previous studies of rhythmic gene expression in the rat pineal (3). Different samples were used for subsequent verification (e.g. Northern) studies. For array, Northern, Western, and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, tissue samples were snap-frozen on dry ice, and stored at -70°C prior to analytical procedures. For *in situ* hybridization analysis, pineals were fixed for 8 h in 10% buffered formalin as described (mRNAlocator[®] kit; Ambion Inc, Austin, TX, USA).

CDNA array analysis

Atlas Rat 1.2 cDNA Expression Arrays (Clontech Laboratories, Palo Alto, CA, USA), consisting of rat cDNA fragments from 1176 genes, were used in this study. Total RNA was extracted from a pool of 2 rat pineals sampled at either 12.00 h (L) or 24.00 h (D) using a standard protocol (16), and DNase I-treated according to the Atlas protocol. RNA quality was then assessed by agarose gel electrophoresis. cDNA probes were synthesized according to the Atlas protocol using a mixture of gene-specific primers which restrict cDNA synthesis to sequences present on the array, thereby reducing background hybridization. Total RNA (2 μg) and primer mixture were denatured at 70°C ; probe synthesis then proceeded at 48°C in 10 μl reactions with the addition of 25 mCi $\alpha^{32}\text{P}$ dATP (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK), 0.5 mM dCTP, 0.5 mM dGTP, 0.5 mM dTTP, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl_2 , 5 mM DTT and 100 U MMLV reverse transcriptase. Labeled probes were purified by column chromatography using the Atlas Nucleospin Extraction Kit, and specific activity measured by liquid scintillation

counting. Radiolabelled probe (1×10^7 c.p.m.) was denatured at 68°C in 0.1 M NaOH and 1 mM EDTA and neutralized by the addition of NaH_2PO_4 (pH 7.0) to a final concentration of 0.5 M. $5 \mu\text{g}$ of C0t-1 DNA was also added. Denatured probe was added to Atlas ExpressHyb hybridization buffer containing 0.1 mg/ml sheared salmon testes DNA, and array hybridizations (day versus night on separate Atlas membranes) were carried out at 68°C in a rotation hybridization oven overnight. Membranes were washed at 68°C for 30 min with continuous agitation in $2 \times \text{SSC}/1\%$ SDS four times, once in $0.1 \times \text{SSC}/0.5\%$ SDS and finally at room temperature in $2 \times \text{SSC}$ for 5 min. Membranes were wrapped in Saran wrap and exposed to a Storage Phosphor screen (Kodak, Rochester, NY, USA) for 3 days. Each membrane was then stripped by boiling in 0.5% SDS and re-probed twice with alternate day and night samples, switching the membrane between each consecutive screen.

A Molecular Imager (FX; BioRad, Hercules, CA, USA) was used to scan exposed phosphor screens at a resolution of 50 microns. Images were visualized using the Quantity One software (BioRad), saved as TIFF files, and imported into the analysis package AtlasImage 1.01 (Clontech). Images were aligned onto an array grid template and day versus night arrays compared. The measured intensity of each gene spot was adjusted by subtracting the background level of intensity, and arrays were additionally normalized according to signal intensities of the glyceraldehyde-3-phosphate dehydrogenase, actin and 40S ribosomal RNA genes which were selected as commonly used 'housekeeping' genes that exhibited both robust expression, and minimal variation between time points. The ratio of the average background adjusted intensity of these three genes between each day and night array constitutes the normalization coefficient. The ratio of normalized and background adjusted signal intensities between specific genes on day versus night arrays was then calculated. Ratios greater than 1.3 or less than 0.77 (2 SD) were considered as significantly different and indicate up, or down-regulation, respectively, of the gene expression at night compared to day. Ratios could not be defined for genes in which the signal is at background level on one array. The difference between signal intensities of such genes were scored visually on a scale of 1–3 indicating increasing differences between signals, and genes with the highest score were considered to be candidates for differential expression.

RNA analysis

Total cellular RNA was extracted, fractionated on formaldehyde-agarose gels, and visualized by Northern analysis as described (16). Probes were labelled by random-priming (17) with ^{32}P -dCTP (Amersham Pharmacia Biotech). Id-1 and Id-3 probes were obtained through RT-PCR amplification as follows. Rat pineal total RNA (1 μg) was reverse transcribed using oligo dT primers (Superscript Preamplification System for First Strand cDNA synthesis, Life Technologies, Paisley, UK). cDNA was then amplified using either the Id1-F1/Id1-R1 or Id3-F/Id3-R primer pairs (sequences obtained from Clontech, Table 1) as described below, with 30 cycles of amplification and an additional final elongation step of 72°C for 10 min. Products were visualized by agarose gel electrophoresis with ethidium bromide staining, the 244 bp (Id-1) and 245 bp (Id-3) bands excised from the gel, purified (QIAEX II Gel extraction kit, Qiagen Ltd, Dorking, UK) and either ligated into the pGEM-T vector (Promega, Southampton, UK) according to the manufacturer's instructions (Id-1) or used directly for ^{32}P -labelling (Id-3). The DNA sequence of the

TABLE 1. Primers for PCR analysis.

Primer name	Primer sequence (5' to 3')	Gene	Location
Id1-F1*	CAT GAA GGT CGC CAG TAG CAG TGC	Id-1	41–65
Id1-R1*	TTT GCG GTT CTG AGG CAG GGT AGG	Id-1	285–261
Id1-F2	ACC CTG CCT CAG AAC CGC AAA	Id-1	264–286
Id1-R2	CCG CGG TAG TGT CTT TCC C	Id-1	834–816
Id1'-R	GAG CTT TTT CCT ATT GGT CGC CC	Id-1	588–565
Id3-F*	CAA CCT CCA ACA TGA AGG CGC TGA G	Id-3	4–29
Id3-R*	GAC GAT GTA GTC TAT GAC ACG CTG CA	Id-3	249–223

*Primer sequences obtained from Clontech Laboratories Inc.

pGEM-T/Id-1 insert was determined (Lark DNA Technologies, Saffron Walden, UK) and found to be 100% homologous to the published rat Id-1 sequence (Genbank no. D10862). Accordingly, a 275-bp *NcoI/NorI* fragment of pGEM-T/Id-1 was used as a probe. Northern blots were stripped (boiling 0.1% SDS) and re-probed with a commercially available 18S cDNA (DecaTemplate, Ambion, Austin, TX, USA). Densitometric analysis of mRNA levels between samples was performed using Imagequant[®] software (3.0, Amersham Pharmacia Biotech), correcting values against the level of 18S RNA.

RT-PCR analysis was performed using the Superscript Preamplification System (Life Technologies) to generate cDNA from rat pineal and retina light and dark phase total RNA in accordance with manufacturer's protocols. Total RNA samples (1 µg) were reverse transcribed using oligo dT primers. Retinal cDNA samples (1 µl) were amplified using the Id1-F1/Id1-R1 or A1/A2 primer pairs (Table 1) in 20 µl reactions containing 20 pmol of each primer, 100 µM dATP, 100 µM dCTP, 100 µM dGTP, 100 µM dTTP, 50 mM KCl, 10 mM Tris-HCl (pH 9) 0.1% Triton (X-100, 1.25 mM MgCl₂ and 0.5 U Taq DNA polymerase (Promega). Thermal cycling was at 94 °C for 3 min followed by 26, 30 or 34 cycles of 95 °C for 45 s, 60 °C (Id1-F1/Id1-R1) or 58 °C (A1/A2) for 45 s, and 72 °C for 45 s. Pineal cDNA samples (1 µl) were analysed for the presence of the Id-1' splice variant using the Id1-F2/Id1'-R (amplifies Id-1' only) and Id1-F2/Id1-R2 (amplifies both Id-1' and Id-1) primer pairs as described above for 20 and 24 cycles. In parallel with the amplification of Id-1 products, aliquots of the same cDNA samples were amplified using an actin cDNA primer pair (18). Amplified cDNA fragments were analysed by agarose gel electrophoresis and compared with a molecular weight marker (1 kb ladder, New England Biolabs, Beverly, MA, USA). All RT-PCR experiments were carried out in duplicate.

In situ hybridization analysis of Id-1 transcript localization in formaldehyde-fixed, paraffin-embedded pineal sections (10 µm), was performed according to the mRNAlocator[®] kit (Ambion) with RNA probes derived from the pGEM-T/Id-1 plasmid described above. Id-1 antisense and sense RNAs were transcribed using SP6 and T7 polymerases, respectively (Riboprobe system (Promega). RNAs were treated with DNase I, purified, spectrophotometrically quantified, and 100 ng of each was labelled with Biotin using the BrightStar soralen-biotin nonisotopic labelling kit (Ambion). Hybridization was performed at 55 °C for 4 h using a 1 : 10 dilution of the *n*-butanol-purified probe in hybridization buffer. Tissue sections were washed (mRNAlocator protocol), and hybridization was detected using the mRNAlocator, Biotin detection kit, employing a 1-h incubation with the NBT/BCIP substrate. Sections were viewed with a Leica DM-RD microscope, and images were captured using a Spot camera (1.30) and Spot Advanced Image software (Spot software 2.2; Diagnostic Instruments, MI, USA), imported into Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA) and rendered grey-scale.

Protein analysis

Western analysis of Id-1 protein expression was performed on whole cell (19) and nuclear (20) pineal extracts as described (16). Protein concentrations were determined by the method of Bradford (21), and mass estimates were obtained with reference to Broad Range Protein Markers (New England Biolabs). The primary antisera used were, Id-1: C-20, sc-488X, (1 : 2000; Santa Cruz Biotechnology Ltd, Santa Cruz, CA, USA), Id-3: C-20, sc-490X (1 : 1000; Santa Cruz Biotechnology Ltd) and Hsp70: 1B5 (1 : 1000; StressGen Biotechnologies Corp., Victoria, BC, Canada). Secondary antisera (Donkey anti-Rabbit IgG-HRP-linked, Amersham Pharmacia Biotech; Sheep anti-mouse IgG-HRP-linked) were both used at a 1 : 5000 final dilution. Proteins were detected using chemiluminescence (HPRL kit, National Diagnostics, Atlanta, GA, USA). The relative level of protein bands were compared using densitometric analysis (Imagequant, Amersham Pharmacia Biotech). Specificity of the Id-1 antiserum was determined by neutralization using a specific Id-1 peptide sequence (sc-488P, Santa Cruz). Aliquots of the antisera were incubated overnight at 4 °C in a 10-fold excess of peptide in PBS, or in PBS alone, and then used to probe Western blots as described above.

Results

CDNA array analysis reveals both novel and known pineal gene rhythms

Array analysis identified 38 genes that exhibited consistent day/night differences in expression (Table 2), according

to criteria for differential expression (22, 23). Differential expression of three of the 38 genes, Id-1, Id-3 and nectadrin (Fig. 1) (24), was confirmed by Northern blot analysis (see below).

In addition to these novel gene rhythms detected in the present study, previously established rhythms were confirmed, including the nocturnal up-regulation of *NGFI-Alegr-1* (9). However, other established pineal mRNA rhythms have not been detected, either because of nonrepresentation of the gene on the cDNA array, or because expression was undetectable with the probes synthesized in the present experimental design.

Nocturnal up-regulation of Id transcript expression in the pineal

The differential day/night expression of two members of the Id family (Id-1 and Id-3) indicated by array analysis (Table 2) was not seen with another member of the Id family, Id-2 (array data not shown). Parallel analysis of Fra-2 target genes in the pineal (24) also identified Id-1 as a potential target (Humphries, Klein, Carter and Baler, unpublished data); accordingly, the Id genes were selected for further analysis. The Id genes are of additional interest in the context of rhythmic expression because, as dominant negative HLH proteins (25), they have a structural, and potentially functional, relationship with the basic helix-loop-helix (bHLH-PAS domain) 'clock' genes which exhibit differential night/day expression in both the rodent pineal gland (26) and the SCN (1).

The differential day/night expression of Id-1 revealed by array analysis was confirmed by northern blot studies, which indicated that there was a six-fold change in the abundance of a single 1.2 kb Id-1 transcript in pineal (Fig. 1A,B). The discrepancy between the six-fold change observed in the northern analysis and the 1.6-fold change in the array analysis (Table 2) reflects the relatively qualitative nature of cDNA array data. A rhythm in a 1.4 kb Id-3 transcript was also confirmed by northern analysis (Fig. 1A) but the amplitude of the rhythm was relatively minor compared with Id-1. The pattern of pineal Id-1 expression conformed to that of other pineal mRNA rhythms, with highest levels at night (Fig. 1C). In addition, the Id-1 mRNA rhythm persisted under conditions of constant darkness (Fig. 1b), indicating that it is generated by an endogenous circadian pacemaker. These findings are consistent with the interpretation that the *Id-1* rhythm is controlled by the retinal to SCN to pineal regulatory pathway (3).

Although a rhythm in Id-1 mRNA was consistently observed in preparations of pineal RNA, a similar rhythm in retinal Id-1 expression was not evident (Figs 1A and 2A). In contrast, a rhythm in Id-3 mRNA was consistently observed in the retina (Fig. 1A).

The relative level of pineal expression of Id-1 and a C-terminal splice variant designated Id-1' was investigated because the different coding potential of Id-1' is associated with different bHLH protein binding specificity (27). Id-1' was found to be expressed in the pineal gland, through visualization of previously characterized RT-PCR amplimers (see 27). However, it was present at markedly lower levels than the

TABLE 2. Genes showing a diurnal rhythm of expression in the rat pineal gland.

Coordinate	GenBank acc. no.	SwissProt acc. no.	Gene name	Ratio	SEM
C13b	D17711	Q07244	dC-stretch binding protein (CSBP); HNRNP K	0.67	0.08
E01a	M13969	Q63371	Insulin like growth factor II (IGF-II)	0.41	0.09
E05i	M89791	P55207	Insulin-like growth factor binding protein 1 precursor	0.56	0.04
F01f	U27767	P08753	Rgs4; regulator of G-protein signalling 4 (RGP4)	0.45	0.18
F13j	X67788	Q63474	Ezrin; cytovillin; villin 2 (VIL2) ; p81	0.30	0.13
F13n	X62908	Q63475	Cofilin	0.65	0.08
A02m	D38492	Q63198	Rat neural adhesion molecule F3	1.52	0.44
A04f	U49062	Q07490	Signal transducer CD24 precursor; nectadrin	6.41	3.50
A06g	D10862	P41135	DNA-binding protein inhibitor ID1	1.60	0.43
A06i	D10864	P41138	Id-3; DNA-binding protein inhibitor; HLH protein	1.48	0.43
A11e	D13374	Q05982	Nm23-M2; nucleoside diphosphate kinase B	1.97	0.53
A11f	M91597	P19804	NDK-B; nucleoside diphosphate kinase B	1.38	0.45
A13k	S45392	P34058	HSP84; HSP90-beta; heat shock 90 kDa protein	1.45	0.17
A14b	U06099	P35704	Thioredoxin peroxidase 1 (TDPX1)	1.95	0.59
B01 h	Y00404	P07632	Copper-zinc-containing superoxide dismutase	1.85	0.74
B09f	M96601	P31643	Taurine transporter	2.85	0.98
B10d	J04629	P13638	Sodium/potassium-transporting ATPase beta 2 subunit	1.68	0.36
B12f	D13123	Q06645	ATP synthase lipid-binding protein P1 precursor;	3.53	1.38
B13l	L38247	P50232	Synaptotagmin IV (SYT4)	*	
B13n	M93669	P10362	Secretogranin II precursor (SGII; SCG2)	2.37	0.69
C01 h	AB003991	P13795	Synaptosomal associated protein 25; SNAP-25	1.35	0.26
C04b	M19044	P10719	Mitochondrial ATP synthase beta precursor (ATP5B)	2.58	0.88
C08n	M27716	P14173	DOPA decarboxylase (DDC)	*	
C10m	D10854	P51635	NADP+ alcohol dehydrogenase; ALR	2.76	0.87
C11f	X14210	P12750	Ribosomal protein S4	1.59	0.59
C14j	L29232	P24062	Insulin-like growth factor I receptor alpha subunit	1.92	0.16
D03d	M84009	P97520	D(4) dopamine receptor; D(2C) dopamine receptor	*	
D08e	J03933	P35370	Thyroid hormone beta receptor; c-erbA-beta	*	
D08l	U17254	P23811	Nur77 early response protein; NGF-I	*	
D09f	L31622	P32301 Q64073	Neuronal acetylcholine receptor protein beta 2 subunit precursor (nonalpha 1; CHRNB2; ACRB2)	1.85	0.26
E06d	M18416	P01143	Early growth response protein 1 (EGR1) ; NGFI-A	1.73	0.16
E08 h	D14592	P29066	Dual-specificity mitogen-activated protein kinase 2	2.49	0.68
E11n	L27843	P19139	Nuclear tyrosine phosphatase; PRL-1;	1.65	0.34
E13e	L29090	P39951	Guanine nucleotide-binding protein G(i) /G(s) /G(t) beta subunit 3 (GNB3)	1.78	0.57
E14i	M83676	O08617	Rab12, ras related GTPase	1.79	0.53
F03e	J04563	P97680	cAMP-dependent 3',5'-cyclic phosphodiesterase 4B	2.68	0.73
F04e	D17615	O08662	14-3-3 protein zeta/delta	1.56	0.30
F04k	M84416	P14270	14-3-3 protein epsilon	1.59	0.39
F10c	D45249	P47820	Proteasome activator rPA28 subunit alpha	1.31	0.46

Genes C13b to F13n and A02m to F10c had higher mRNA levels in the light and dark phases, respectively. *Ratios cannot be calculated as the level of intensity during the day was nearing or equal to the background signal. The data represent array experiments carried out in triplicate.

alternative Id-1 transcript and does not exhibit a different pattern (relative to Id-1) of rhythmic expression (Fig. 2B). Thus, nocturnal up-regulation of the Id-1 gene appears to be primarily manifested at the transcriptional level, rather than involving splice-variant-selective, post-transcriptional control.

Distribution of Id-1 transcript in the pineal

In situ hybridization indicated that Id-1 was expressed within the cytoplasm of the major cell type in the pineal gland (Fig. 3), the regularly shaped melatonin-producing pinealocyte.

Id-1 protein exhibits a circadian rhythm of expression in the pineal

The expression and regulation of Id-1 protein in the rat pineal gland was studied (Fig. 4). Multiple protein bands were

observed on standard SDS-PAGE resolved Western blots (Fig. 4A), in agreement with published reports with other tissue preparations (28). All bands represent a specific immunoreaction because the signals were blocked by preadsorption of the antibody (C-20) with Id-1 peptide (data not shown) or with recombinant full-length Id-1 protein (28). The pattern of immunopositive bands comprised a monomeric (18–20 kDa), and larger multimeric or complexed forms. The larger forms are thought to represent in part complexes containing Id-1 tightly bound to other bHLH proteins (28).

The monomeric form of Id-1 (18–20 kDa) exhibited an approximately six-fold night/day rhythm (Fig. 4A, upper), similar to the rhythm in Id-1 mRNA levels (see above). This parallel relationship suggests that the 18–20 kDa protein rhythm is driven by the mRNA rhythm and that the Id-1 monomer turns over rapidly, suggesting a night-specific function. The 50–55 kDa Id-1 immunoreactive band also

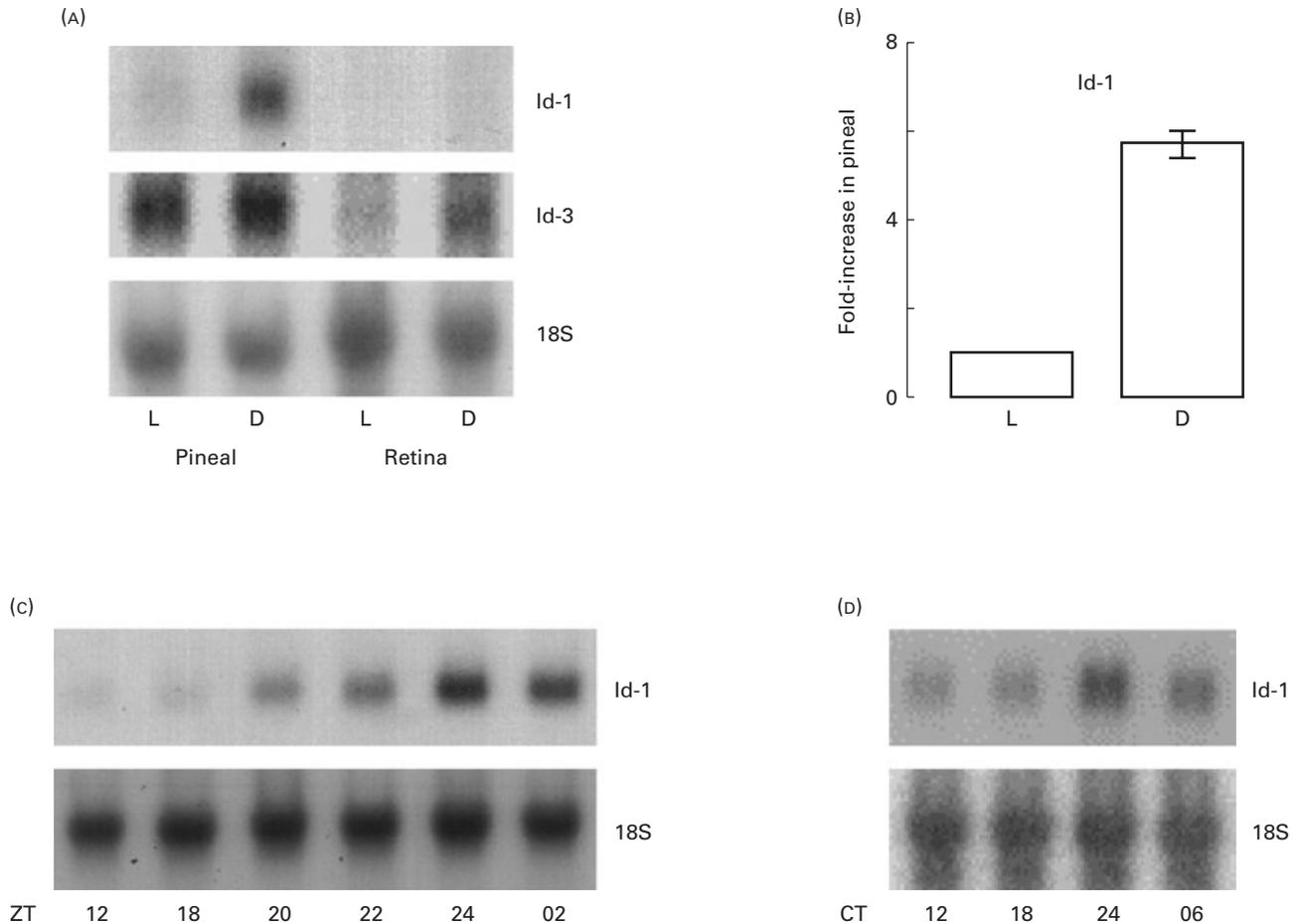


FIG. 1. Circadian rhythm of Id-1 mRNA expression in the pineal gland. Northern analysis of total cellular RNA (A) 7 $\mu\text{g}/\text{lane}$, (C) 5 $\mu\text{g}/\text{lane}$, (D) 10 $\mu\text{g}/\text{lane}$, extracted from the pineal gland or retina of rats hybridized, first, with a ^{32}P -labelled, Id-1 cDNA probe, second, with a ^{32}P -labelled, 18S probe and, third, (A) only with a ^{32}P -labelled, Id-3 cDNA probe (A) Representative northern analysis of RNA extracted from rats were killed at either 12.00 h (L) or 24.00 h (D). Exposure times: Id-1, 3 days (X-ray); Id-3, 1 day (Phosphor screen); 18S, 15 min (X-ray). (B) Quantitative analysis of multiple pineal samples ($n=3$ pairs in each group) analysed as in (A). The levels of Id-1 mRNA were normalized against the levels of 18S RNA and are presented as fold-change compared with the Light phase (L) levels. Values are mean \pm SEM. (C) Representative northern analysis of RNA extracted from paired rats killed at different times of the L:D cycle relative to lights out at 19.00 h. Exposure times: Id-1, 4 days; 18S, 10 min. (D) Representative northern analysis of RNA extracted from paired rats killed at different times during constant darkness. Exposure times: Id-1, 4 days; 18S, 15 min.

exhibited a night/day rhythm. Interestingly, this band is more highly represented within the nuclear fraction (Fig. 4, lower). Densitometric analysis of this band revealed that it represented 27% of the total Id-1 immunoreactive protein. In contrast to the rhythmic expression of the 50–55 kDa nuclear Id-1 band, the smaller 20 kDa Id-1 form does not exhibit a marked nuclear rhythm indicating that the monomer may be primarily restricted to a cytoplasmic pool. High molecular weight bands of approximately 83 kDa and 150 kDa in the pineal gland did not exhibit rhythmic changes; in contrast, dynamic changes in these signals have been reported to occur in other tissues (28)

Analysis of Id-3 protein expression in whole cell extracts revealed an (Id-1-like) complex pattern presumably also reflecting the presence of monomeric and complexed forms (data not shown). However, none of the Id-3 forms, including the monomeric form (Fig. 4B, middle), exhibited any evidence of rhythmic variation; in contrast, day/night changes in

Id-1 described above were detected in this preparation (Fig. 4B, upper).

The indication that expression of the Id-1 gene was circadian in nature was supported by analysis of Id-1 protein; this revealed that the rhythm in the 18–20 kDa form persisted in a constant environment. Thus, a comparison of the Id-1 (20 kDa) protein level at 24.00 h between DD and LD sampled rats revealed that the level at CT 24 sampled in DD was $87 \pm 16\%$ of the level at ZT 24 sampled in LD 14:10 ($n=3$ pairs of pineals in each group).

Discussion

The current study has demonstrated the utility of cDNA array analysis for the identification of novel gene rhythms within circadian systems, in that multiple potential new rhythms have been identified. Of these, four have been confirmed using Northern analysis of transcript expression: Id-1 and Id-3

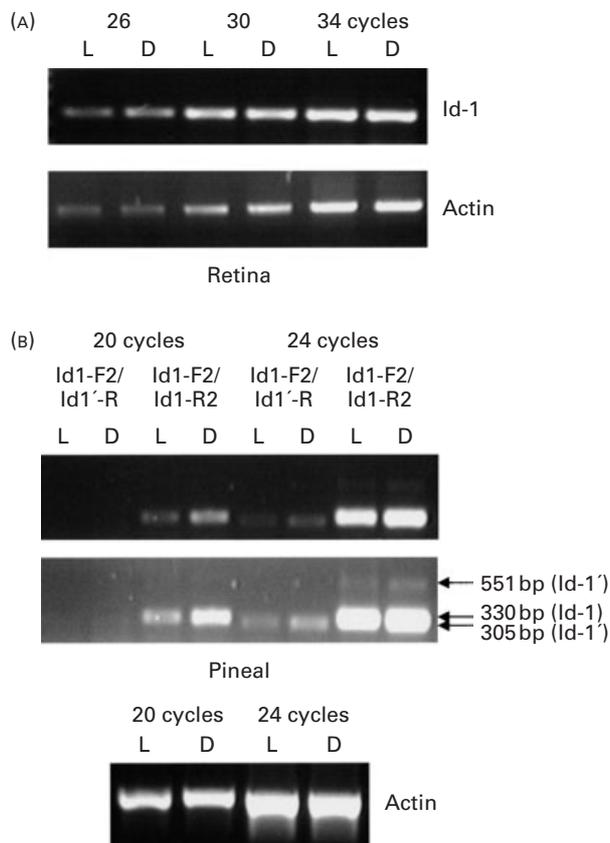


FIG. 2. Absence of both Id-1 mRNA rhythm in the retina, and differential Id-1 splice variant rhythm in the pineal gland. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of total cellular RNA extracted from the pineal gland and retina of rats killed at either 12.00 h (L) or 24.00 h (D), using primers designed to specifically amplify either a single 244 bp Id-1 cDNA fragment (A), or multiple Id-1 and Id-1 splice variant (Id-1') fragments (B). Amplified fragments were resolved on 1% agarose gels and visualized using ethidium bromide staining. (A) Representative analysis of retinal Id-1 mRNA expression at different PCR cycle numbers compared to amplification of actin mRNA. (B) Upper panels: representative analysis of pineal Id-1 and Id-1' mRNA expression showing amplification of Id-1 (330 bp) and Id-1' (305 and 551 bp) specific cDNA fragments at different PCR cycle numbers. The two panels represent different exposure times of the camera used to capture the images, and are shown for clarity. Lower panel: representative analysis of actin mRNA expression using the same samples as in the upper Id-1/Id-1' analysis.

(present study), nectadrin (24) and NGFI-A (9). Clearly, our findings are only partially representative of the full complement of rhythmically expressed genes within the mammalian pineal; many other gene rhythms have been identified previously using both differential screening (13–15) and conventional approaches (7–10). It should also be noted that the design of the current screen will select for genes that exhibit either prolonged nocturnal up- or down-regulation, or alternatively changes in expression during the plateau phase of nocturnal melatonin secretion, rather than transient changes during the ascending or descending phases. For example, some previously identified gene rhythms such as the transient increase in *jun-B* expression 2 h after the onset of darkness (10) would not be detected in the current screen. Therefore, future

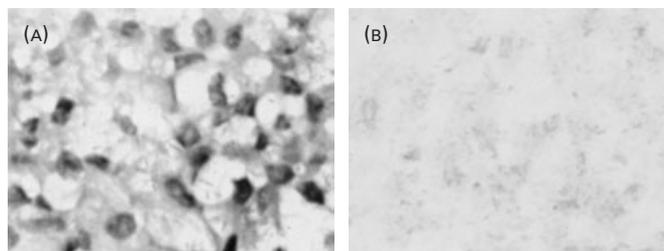


FIG. 3. Id-1 mRNA is expressed in pinealocytes. (A,B). *In situ* hybridization analysis of Id-1 mRNA expression in pineal glands of rats killed at 24.00 h using antisense (A) and sense (B) biotin-labelled probes. Hybridization was visualized using the NBT/BCIP substrate which generates a blue colour reaction but the images have been rendered greyscale. Note the presence of a strong hybridization signal in (A) only, within the cytoplasm of a major population of regularly shaped cells. Similar images were observed in multiple sections derived from two pineal glands. Magnification $\times 400$.

application of this approach using a more extensive series of RNA sample points would be expected to reveal additional genes which are differentially expressed on a circadian basis. Given the partial transcriptome level of our current cDNA array analysis, our data are therefore suggestive of a multitude of rhythmically expressed genes in the rat pineal, and extensive functional genomic (e.g. transgenic) studies will be required to address the physiological importance of these rhythms.

The discovery in the current study of a circadian rhythm in the expression of Id-1 in the melatonin-secreting pinealocytes suggests that Id-1 may be involved in the rhythm in AANAT expression (6). The lower level of Id-1 expression in the retina could be considered to be consistent with the low levels of AANAT expression in this tissue (3).

Previous studies of the Id genes have focused on their developmental role within differentiation (29, 30) and potential oncogenic role within tumorigenesis (31–33). The Id genes are HLH proteins, but are distinct from the major bHLH class of transcription factors because they lack the basic DNA binding domain. These proteins have therefore been recognized as naturally occurring dominant negative regulators which can inhibit the function of bHLH factors through the competitive formation of Id-bHLH heterodimers (in preference to Id homodimers) which are then excluded from DNA interaction (30). Id-1 was originally identified in 1990 (25) and has been shown to participate in cellular regulation through changes in expression level (34, 35), suggesting that the rhythm of expression observed in the present study may be functionally relevant. It should not be assumed, however, that the potential role of Id-1 is strictly limited to a dominant negative interaction because in, recent recombinant fusion protein studies (36), Id proteins have been shown to activate transcription when linked with the Gal4 DNA-binding domain. Whether Id-1 can associate into similar functional complexes within cells is unknown.

The nocturnal up-regulation of Id-1 gene expression observed here (sixfold) is similar in magnitude to the previously observed induction of Id-1 by NGF in undifferentiated rat neural cells (eightfold) (37). Our quantitative analysis therefore suggests that the rhythmic expression of Id-1 in the pineal is functionally meaningful in so much as it resembles

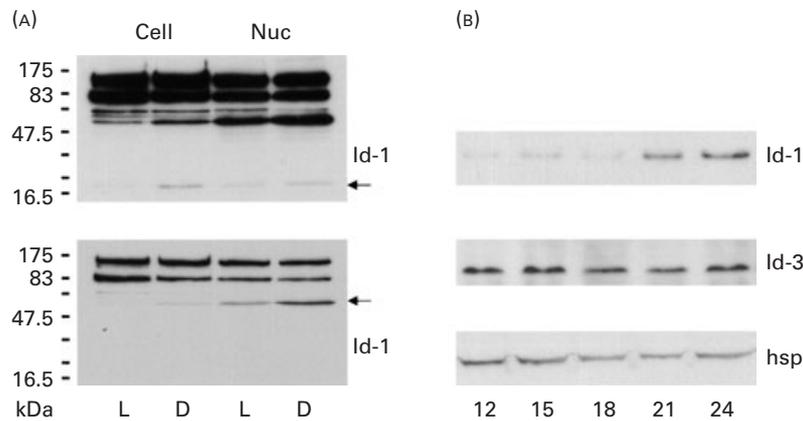


FIG. 4. Day: night rhythm of Id-1, but not Id-3, protein expression in the pineal gland. Western blot analysis of either whole cell (cell) or nuclear (nuc) protein fractions extracted from the pineal gland, resolved on SDS-PAGE gels, probed with specific antisera, and visualized using chemiluminescence. (A) Representative analysis of Id-1 expression in pineal glands sampled at either 12.00 h (L) or 24.00 h (D). Different exposures of the same blot are shown for clarity: upper, 25 min; lower, 15 min. Horizontal bars on the left represent molecular mass markers, and arrows on the right indicate the position of 20 kDa (upper panel) and 50 kDa (lower panel) protein bands. (B) Representative analysis of Id-1 expression in pineal glands sampled at different times of the L:D cycle relative to lights out at 19.00 h. Note that only the monomeric forms of Id-1 and Id-3 are shown for clarity. hsp, heat shock protein-70.

the induction profile observed during early neural differentiation events. However, in contrast to the NGF response, which is inclusive of Id-1, Id-2 and Id-3 (37), we have shown that Id-2 does not exhibit rhythmic pineal expression, and the Id-3 mRNA rhythm was not manifested at the protein level. A gene-specific role for Id-1 in nocturnal pineal function is therefore indicated, a finding consistent with other recent studies which have provided evidence of specific roles for each Id gene (38–40).

As discussed above, the primary identified functional activity of the Id proteins is as negatively acting transcriptional regulators which can modulate the DNA binding activity of bHLH *trans*-acting factors. Our demonstration that the nocturnal induction of Id-1 in the pineal is associated with an increase in the assembly of a presumed heteromeric nuclear protein complex implicates an associated change in E-box DNA binding activity. However, our immunoblotting analysis has revealed that, although the rhythmic fraction of the nuclear Id-1-immunoreactive pool represents as much as 27% of the total pool at night, there remains an abundance of a nonrhythmic fraction of complexed Id-1 protein. Consequently, a (rhythmic) Id-1-dependent change in DNA binding activity would be restricted to a particular subset of E-box binding proteins. Because the formation of cell type-specific E-box binding complexes is crucially dependent upon the identity of flanking sequences which surround the consensus hexamer sequence (41, 42), a protein interaction library screening approach may be required to identify rhythmic Id-1 targets in the pineal. A recent report of rhythmic expression of the bHLH-PAS domain genes *per1* and *per2* in the pineal (26) has indicated two potential targets, but it is not clear whether these gene products can interact with Id proteins. At the same time, it should be noted that potential Id-1-interacting proteins need not be rhythmically expressed. Clearly, an understanding of the role of the Id-1 rhythm within the pineal must await the identification of specific targets.

In conclusion, our demonstration of a precisely timed rhythm of Id-1 expression within the pineal gland has

provided novel evidence of a role for this HLH protein within circadian function. This finding is in accordance with a central role for transcriptional regulators in biological timing mechanisms, but implicates a novel mode of regulation involving dominant negative factors. Our establishment of this model of Id-1 induction has also provided an experimental paradigm through which Id-1-specific target proteins can be identified.

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