Indication of circadian oscillations in the rat pancreas

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Abstract The central circadian oscillator of the suprachiasmatic nucleus controls diurnal rhythmicity of the body with light as its dominant zeitgeber. Recently, peripheral oscillators have been detected in liver and heart, which follow as yet unidentified cues. In this study real-time reverse transcription-polymerase chain reaction (RT-PCR) was used in analysis of the expression of the major clock genes *Per1*, *Per2*, *Bmal1*, *Cry1*, *Tim* (*timeless*) and *Clock*, as well as of the output genes *Dbp* and *Rev-erbα* in the pancreatic tissue of rats. The results presented here indicate a robust circadian expression of clock genes (e.g. *Per1* and *Bmal1*) and the probable existence of a peripheral oscillator in the pancreas. Whether this oscillator regulates the diverse functions of the islets of Langerhans remains to be elucidated.

Key words: Insulin secretion; Clock gene; Circadian expression; Rat pancreatic islet; Real-time reverse transcription-polymerase chain reaction

1. Introduction

In mammals the central circadian pacemaker of the suprachiasmatic nucleus (SCN) comprises two intertwining feedback loops, with BMAL/CLOCK as the driving components of the positive limb and CRY/PER heterodimers as components of the negative limb. The genes of CRY/PER are activated by BMAL/CLOCK and repress this activation at the end of each circadian cycle [1,2]. Light is the zeitgeber for the central circadian oscillator (CCO), which couples internal rhythms to the objective day/night patterns [3,4].

Recent research, however, indicates that, besides the central clock of the brain, peripheral oscillators exist in several organs, such as the heart [5,6], liver [7,8], kidney [9], oviduct [10] and hypophyseal *pars tuberalis* [11]. Differences in circadian phase distribution suggest tissue-specific circadian clock regulation. There is evidence that a circadian oscillator could even function on a cellular level [12–14]. In contrast to the CCO, factors other than light entrain the rhythm of peripheral organs which can be uncoupled from the 'master clock', for example, by restricted feeding [7,15]. These factors are as yet unknown; however, glucocorticoids [7,16], glucose [14], melatonin [17] and other humoral factors, probably specific for each organ, entrain tissue-specific oscillations. Besides the central, activity-dominating rhythm generator of the SCN, 'peripheral clocks', e.g. in the liver and pancreas, are evolutionarily conserved mechanisms, which enable the organisms to anticipate, for example, changes in glucose levels due to food intake [18]. Whether disturbances of pancreatic oscillations are part of the complex events leading to diabetes is an open question, but the onset of diabetes mellitus has been associated with a loss of diurnal rhythmicity in insulin secretion [19].

Peschke and Peschke [17] determined that insulin from isolated rat islets is released in a circadian manner (τ between 22 and 26 h) and is influenced by melatonin as a zeitgeber. Delattre et al. [19] speculated that adenosine triphosphate (ATP) oscillations might be the underlying mechanism for circadian insulin release. In order to elucidate the basis for pancreatic circadian oscillations we studied changes in the expression of the following clock genes on the transcriptional level: *Clock*, *Per1*, *Per2*, *Cry1*, *Tim* and *Bmal1* during a 24-h period by real-time reverse transcription-polymerase chain reaction (RT-PCR). In addition, circadian transcript changes of the clock-controlled output genes *Dbp* and *Rev-erbα* [20,21] were monitored. The findings of this study suggest the function of a circadian pacemaker in the rat pancreas. Furthermore, we show evidence for a circadian oscillator in the islets.

2. Materials and methods

2.1. Animals and cells

Male Wistar rats (age: 44 days) were caged individually and subjected to a lighting regime of light:dark = 12 h:12 h, light on: 6:00 a.m. Rats were fed a standard diet ad libitum. Four animals per time point were killed in a deep anesthesia at 3-h intervals. Tissue sampling proceeded under red light during the dark phase. Animals were treated according to German animal welfare regulations throughout the sampling procedure.

For high-serum treatment, INS1 rat insulinoma β-cells (kindly provided by C.B. Wollheim, University Medical Center, Geneva, Switzerland) were incubated with 50% horse serum in RPMI 1640 medium (Biochrom KG, Berlin, Germany) for 1 h after preincubation of the cells in serum-free medium overnight. Cell sampling proceeded 20 h after serum shock treatment.

2.2. RNA extraction and RT

RNA was extracted from pancreata immersed in RNAlater (Ambion Inc., TX, USA) immediately after killing, using a Trizol-based extraction method according to the manufacturer's instructions (Invitrogen, CA, USA). To extract RNA from the islets, they were separated from exocrine tissue by collagenase treatment and collected as described by Peschke and Peschke [17]. Exocrine tissue cells and islets were extracted immediately. Total RNA concentrations were determined by spectrophotometry at 260 nm, and total RNA quality was assessed by electrophoresis on 1.3% denaturing formaldehyde agarose

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Abbreviations: PCR, polymerase chain reaction; CT, circadian time

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Table 1

Name	Primer sequence		Primer site	Product length (bp)	Citation	GenBank acc. no.
Clock	forw.	5′-AAGTTAGGGCTGAAAGACGGCG-3′	1139–1160	173	[33], modified	NM021856
	rev.	5′-TGAGCTTCTGGAGGAGGCAGAAG-3′	1289–1311			
Per-1	forw.	5′-CCAGGCCCGGAGAACCTTTTT-3′	41–60	403	[34]	AB002108
	rev.	5′-CGAAGTTTGAGCTCCCGAAGT-3′	421–442			
Per-2	forw.	5′-GCAGCCTTTCGATTATTCTC-3′	1267–1287	97	[5]	AB016532
	rev.	5′-GCTCCACGGGTTGATGAAG-3′	1345–1364			
Bmal1	forw.	5′-AGATTGAAAAGAGGCGTCGGGA-3′	642–663	382	[35], modified	NM007489
	rev.	5′-TCCTTAACTTTGGCAATATC-3′	1004–1023			
Cry-1	forw	5′-AGTTCCCCTCCCCTTTCTCTT-3′	1156–1176	101	[36]	AF156986
	rev.	5′-GGGTTCCCTTCCATTTTGTCA-3′	1236–1256			
Dbp	forw.	5′-CCGTGGAGGTGCTAATGACCT-3′	960–980	105	[36]	J03179
	rev.	5′-CCTCTGAGAAGCGGTGCCT-3′	1046–1064			
β-Actin	forw.	5′-ACTCCTACGTGGGCGACGAGG-3′	152–172	389	(U. Musshoff, n.p.)	NM031144
	rev.	5′-CAGGTCCAGACGCAGGATGGC-3′	520–540			
Tim	forw.	5′-GGAGAAAGCTCAGCAACATGATGA-3′	1206–1229	164	[33] modified	NM031340
	rev.	5′-TCAGCTCATAGTAGTTGGTGAGG-3′	1347–136			
Rev-erbα	forw.	5′-ACAGCTGACACCACCCAGATC-3′	516–536	101	[10]	M25804
	rev.	5′-CATGGGCATAGGTGAAGATTTCT-3′	594–616			

n.p. = not published.

gels. For the elimination of residual DNA, total RNA was subsequently subjected to DNase 1 digestion (DNA-*free*™, Ambion Inc., TX, USA). Total RNA (1 μg) was reverse transcribed using an RT kit from Promega (Promega Inc., WI, USA) according to the manufacturer's protocols.

2.3. Real-time RT-PCR

For real-time RT-PCR, 40 ng of cDNA was used per reaction. Primers and sequences are listed in Table 1. Primers were intron spanning with the exception of *Per2*, *Dbp* and *Rev-erbα* primers. Each reaction volume of 20 μl contained 10 μl of a reaction mix (Promega Inc., WI, USA), 0.5 μl of each primer (25 pmol/μl), 0.5 μl of Sybr green I at 10^3-fold dilution (Biozym Diagnostic GmbH, Hessisch-Oldendorf, Germany) and 4.5 μl of H_2O. PCR was performed using a rotor cycler (Rotor-Gene 2000, Corbett Research Inc.; Mortlake, NSW, Australia) and the following thermoprofile: initial denaturation at 95°C for 120 s, 40 cycles with 30 s denaturation at 94°C, 30 s annealing at 59°C (touchdown from 64°C with a 1°C decrease per cycle) and 30 s elongation at 72°C. At the end of each elongation step fluorescence was determined for 15 s at a temperature of 80°C. For amplicon analysis a thermal denaturation profile was generated after each run. The PCR products were further analyzed by separation on Tris–acetate–ethylenediamine tetraacetic acid (EDTA) buffer (TAE)-buffered 3% agarose gels (pH 8.0) and subsequent ethidium bromide staining. Quantification of mRNA was achieved using Corbett Research software, comparing fluorescence intensities at a threshold level (C_t values) within the logarithmic range of the amplification curve [22]. *β-Actin* expression was used for normalization of target gene values throughout the experiments. Reaction efficiency was between 0.80 and 0.92 for target gene amplification, and 0.82 for the housekeeping gene *β-Actin*. Efficiency was calculated for each primer set from runs with serial 10-fold cDNA dilutions by using Corbett Research software. The levels of *β-Actin* mRNA in the pancreas did not vary significantly as a function of time of day in accordance with observations of Damiola et al. [15]. Identity of the PCR products was verified by sequencing on a Perkin Elmer ABI 377 sequencer (Perkin Elmer, Applied Biosystems Inc., CT, USA). Control PCRs were performed using PCR reaction mix including primers without cDNA (non-template control) or with 1 μg of total RNA in the reaction mixture (control for DNA carryover).

2.4. Western blot

Western blots were performed as described by Mühlbauer et al. [23] using 25 μg of total pancreatic protein per lane. Polyclonal anti-mouse Per1 antibody (Per1 PA1–524) was obtained from ABR (Affinity BioReagents, CO, USA) and used at a 1:1000 dilution. For pre-absorption control experiments, testing signal specificity, the peptide with the original antibody epitope SLADDTDANSNG (ABR-PEP-076-C050; ABR, CO, USA) was used as described by the manufacturer.

2.5. Statistical analysis

For statistical evaluation of results and significance testing of group differences, the non-parametric Mann–Whitney *U*-test was performed using GraphPad software (GraphPad Software Inc., CA, USA). Groups were considered to be significantly different at $P < 0.05$. Data are presented as means ± S.E.M. of four animals per group unless otherwise indicated.

3. Results

3.1. Real-time RT-PCR

Analysis of the expression of circadian clock components indicates circadian oscillation of *Per1* mRNA (Fig. 1A). A maximum of expression was observed at circadian time (CT) 11 (5:00 p.m.). The expression level was 13 times higher than that of CT 2, the time of minimum expression. *Per2* mRNA analysis demonstrates low-level expression from CT 2 to CT 8, followed by a strong (approximately 4.3-fold) increase in expression at CT 11, and with a high level of expression throughout the night times (Fig. 1B). *Cry1*, as one of the predominant effectors of the negative limb of the circadian cycle, was expressed at a low level between CT 2 and CT 8, followed by an increase during the late day and at night, with a 10-fold higher level at CT 23 compared to the minimum at CT 8 (Fig. 1C). *Bmal1*, determining the positive part of the feedback loop (together with *Clock*) of the circadian cycle, was found to be expressed reverse to *Per1*. A minimum *Bmal1* transcript level was measured at CT 14 and two maxima at CT 2 and CT 23 (Fig. 1D), thus forming a trough-shaped curve. We did not find evidence for cyclic expression of *Clock* or *Tim* (*timeless*), however, in rat pancreas (Fig. 1E and F). Both genes thus appear either to be constitutively expressed in the pancreas or to oscillate with a very low amplitude below statistical significance.

One of the output genes of the pancreatic oscillator, *Dbp* (Fig. 2A), revealed an expression profile in parallel to *Per1*, with an expression maximum at CT 11 and a 28-fold higher expression level compared to the minimum at CT 2. A similar profile, but with an even higher amplitude (46-fold difference between minimum expression at CT 2 and the maximum at CT 11), was observed for Rev-erbα, a member of the nuclear orphan receptor family (Fig. 2B) [24].

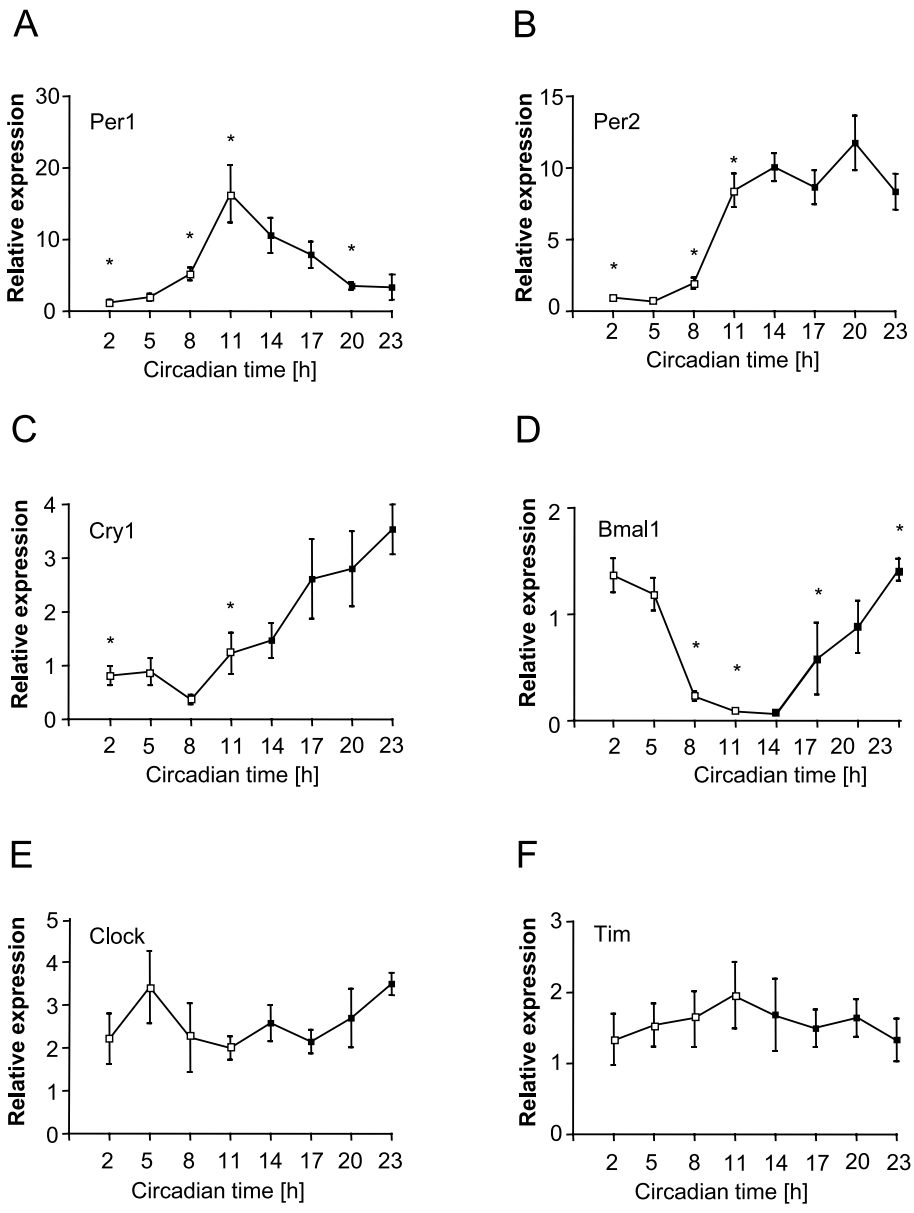

Fig. 1. Circadian expression patterns of clock genes in the rat pancreas. Quantitative real-time RT-PCR results of four animals per time point (unless otherwise stated). Data are expressed as means ± S.E.M. CT 0 was defined as light on. A: Circadian changes of *Per1* mRNA abundance. B: Circadian oscillation of *Per2* mRNA. C: The circadian expression pattern of the clock gene *Cry1*. D: The *Bmal1* mRNA accumulation pattern is opposite that of *Per1* transcripts in rat pancreas ($n = 6$ animals for time points 2, 5 and 23). E, F: The transcripts of the clock genes *Clock* and *Tim* in rat pancreas are not influenced by the time of the day. Statistical analysis was performed by Mann–Whitney *U*-test. Open squares: time points with light on; filled squares: time points with light off; asterisks: statistical differences between time points at $P < 0.05$.

Besides whole-organ pancreatic extracts, clock gene expression is detectable in isolated pancreatic islets, exocrine tissue and INS1 rat insulinoma cells, as indicated by Fig. 3. Hypothalamic expression served as a positive control. Gel electrophoretic separation of RT-PCR products revealed specific transcript occurrence in pancreatic islets and exocrine tissue as well as β-cells (INS1 insulinoma cells). The output genes *Dbp* and Rev-erbα were expressed in all cell types as well; specific PCR products were detected in islets, exocrine tissue and INS1 cells (Fig. 3, bottom).

3.2. Western blot experiments

PER1 expression was confirmed by Western blot experiments detecting a specific major band at approximately 45 kDa in high serum-treated INS1 cells, which was suppressible after incubation of the antibody with the peptide against which the antibody was raised (+P, Fig. 2C, left). Bands of similar molecular weight appeared in rat (R) and mouse (M) pancreatic tissue which was described as the nuclear isoform of the protein by Chilov et al. ([25], Fig. 2C, middle) who used the same N-terminus-targeted antibody for PER1 detection in liver and kidney. They also detected a 55-kDa isoform in these organs which might correspond with the higher molecular weight weak band of similar molecular size in INS1 cells (Fig. 2C, left). The pancreatic signal could be suppressed in pre-absorption experiments with the epitope (Fig. 2C, right).

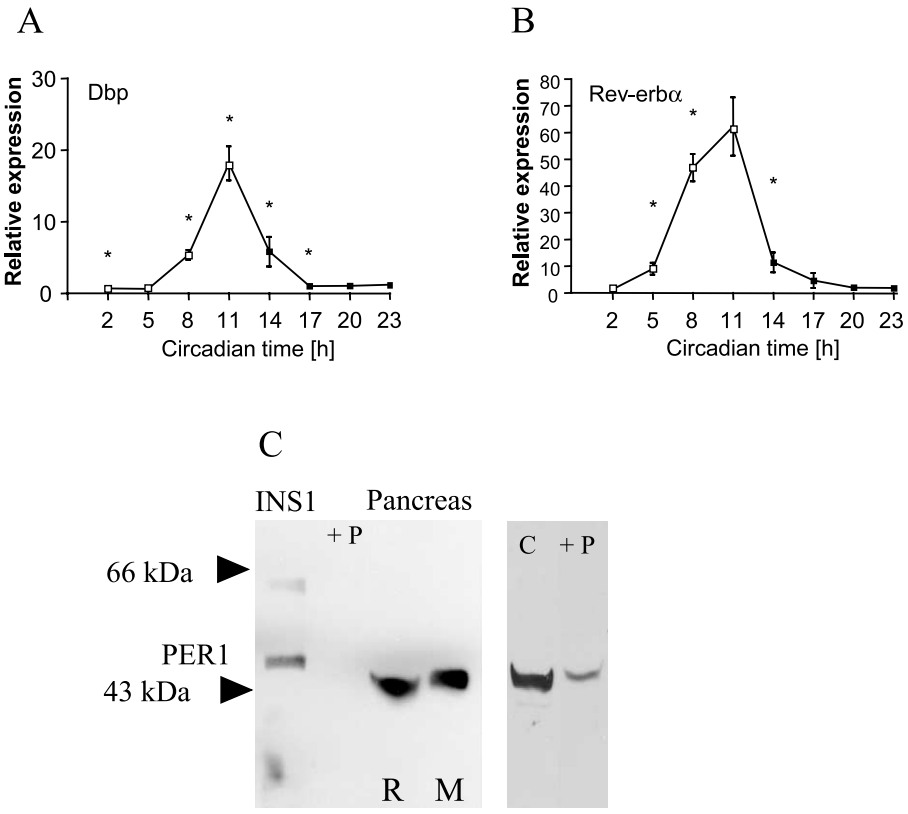

Fig. 2. Circadian expression patterns of the clock-controlled output genes *Dbp* and *Rev-erbα*. Quantitative real-time RT-PCR results of four animals per time point. All data are expressed as means ± S.E.M. A: Circadian oscillation of *Dbp*, with maximum expression at CT 11. B: *Rev-erbα* expression is parallel to that of *Dbp* (and *Per1*) in the rat pancreas, however, with a very high amplitude and a transcript peak at CT 11. Statistical analysis was performed by Mann–Whitney *U*-test (open squares: time points with light on; filled squares: time points with light off; asterisks: statistical differences between time points at $P < 0.05$). C: Western blot detection of PER1 in high serum-treated INS1 rat insulinoma β-cells. Left lanes indicate occurrence of a major band at ~45 kDa, which was suppressible in a pre-absorption experiment by control peptide with the epitope (+P). Western blot detection of PER1 protein in pancreatic tissues of rat (R) and mouse (M; middle lanes) demonstrates the existence of a major band at approximately 45 kDa; this likely signifies the presumed nuclear form of PER1 [25]. Pre-absorption experiments with control peptide demonstrate signal suppression (right lanes: control (C); pre-absorption (+P)).

4. Discussion

Rhythmic insulin release from pancreatic β-cells is a well-known phenomenon [26]. Diurnal rhythmicity of islets has been suggested to play a role in insulin secretion [27] and has been proven to occur in isolated rat islets [17,19]. The latter authors speculated that the circadian oscillator might regulate insulin release via neural signaling, a concept based on the earlier work of Sakaguchi et al. [28]. The data of Peschke and Peschke on rat islets [17], however, pointed out the existence of an islet-located circadian pacemaker. The present study was aimed at elucidating the molecular mechanisms of the putative pancreatic oscillator. To this end we have analyzed clock gene transcript levels of the six major factors: *Clock*, *Per1*, *Per2*, *Tim*, *Bmal1* and *Cry1* during a 24-h period. Changes of expression levels, particularly of *Per1* and *Bmal1* but also of *Per2*, signal the existence of a circadian oscillator in the rat pancreas.

The presence of PER1 in rat and murine pancreatic tissues as a ~45-kDa protein indicates a translation product of low molecular size. This protein is of the same molecular size as the PER1 identified in liver and brain by Chilov et al. [25] who presumed this to be a nuclear form. In INS1 insulinoma cells, PER1 was detected as a major band in serum-treated cells at approximately 45 kDa, together with a weak band at approximately 60 kDa; both were suppressible by the control peptide. As originally observed by Balsalobre et al. [12] serum shock leads to induction of clock genes. We assume that the roughly 45-kDa-sized major band also represents the nuclear form in INS1 cells, in accordance with the predominant 45-kDa form which is found in pancreatic tissue. The apparently larger protein in INS1 cells compared to that in pancreas cells might be due to differences in phosphorylation leading to a greater molecular mass in the β-cell line [29].

The characteristic antiphase expression profiles of *Per1* and *Bmal1*, which were described for the SCN [2] but also for peripheral tissues [9,5] were also characteristic of the pancreatic oscillator in this study. The *Per1* transcript maximum at CT 11 largely overlaps with trough values for *Bmal1* expression at CT 11 and CT 14. This observation is in accordance with the proposed functions of the PAS transcription factors PER1 and BMAL1 within their respective feedback loops [2,21]. *Clock* and *Tim* did not show a clear circadian rhythm in the pancreas which is in accord with results of others [4], although Young et al. [5] and Preitner et al. [21] reported a low-amplitude circadian rhythmicity for *Clock* in heart and liver, respectively. *Cry1* transcripts, the translation products of which are known to be the dimerization partner of PER, were reported to be rhythmic in the SCN [1] and heart, although with low amplitude in the heart [6] and seem to be

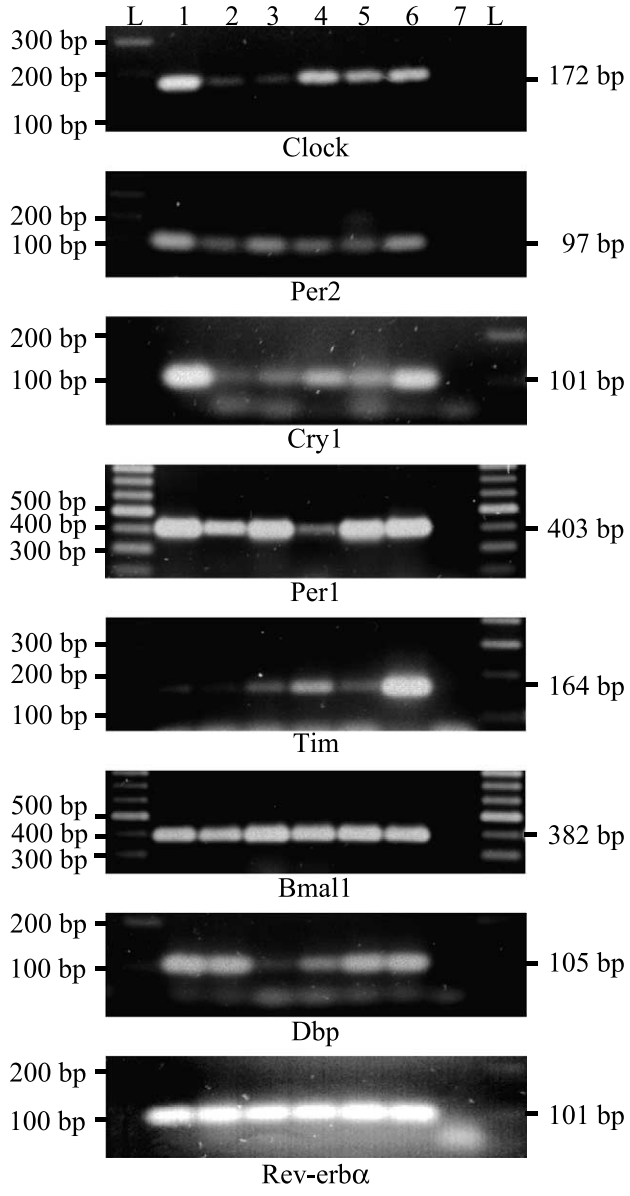

Fig. 3. Expression pattern of clock and clock-controlled genes in pancreatic and subpancreatic rat tissue. RT-PCR of transcripts from rat pancreas, islets and exocrine tissues and of the β-cell insulinoma cell line INS1. Gel electrophoresis of PCR products and subsequent staining of bands with ethidium bromide demonstrates that the clock genes *Clock*, *Per2*, *Cry1*, *Per1*, *Tim* and *Bmal1* as well as the clock output genes *Dbp* and *Rev-erbα* are expressed in pancreatic islets and INS1 β-cells (bands: 1, hypothalamus; 2, pancreas (day); 3, pancreas (night); 4, pancreatic islets; 5, exocrine pancreas; 6, INS1 insulinoma β-cells; 7, non-template (H_2O) control). Molecular sizes of the respective specific PCR products in relation to a molecular size standard (L) are indicated.

rhythmic in the pancreas as well according to our results. The *Cry1* and *Per2* expression maxima at night were also observed by Preitner et al. in the mouse liver [21] – a very similar pattern as detected in the pancreas. One of the clock-controlled output genes, namely the transcription factor *Dbp* [20], was found to oscillate in rat pancreas with a large amplitude, in accordance with previous results from others on liver, kidney, muscle, lung [15,9] and heart [5]. Confirming our results, a study on mice SCN from Yamaguchi et al. [30] describes *Dbp* oscillation to be in phase with *Per1* expression. These authors also propose a role for *Dpb* within the circadian core clock mechanism besides its function as an output recipient. Damiola et al. [15] have determined circadian *Dbp* expression in whole pancreas extracts and assumed food to be the dominant zeitgeber for this organ, as well as for the liver. Our data, which indicate expression of *Dbp* within the islet of Langerhans and INS1 cells, also indicate a functional output from an endogenous islet-based oscillator and substantiate the observed cyclic pattern of insulin release from isolated islets [17]. The orphan receptor *Rev-erbα*, which generally acts as a transcriptional repressor, is known to be rhythmically expressed in the SCN and liver as target of BMAL1/Clock-mediated activation [21]. *Rev-erbα* transcript quantity also varies with a high amplitude in the rat pancreas according to our data. REV-ERBα, which is supposedly a major circadian regulator of *Bmal1* expression, is concomitantly the recipient of the PER-mediated negative output [21]. In accordance with this assumption *Bmal1* transcripts are at a minimum in the pancreas when the *Rev-erbα* transcription level is at its maximum.

In contrast to the CCO in the SCN, the amplitude of the peripheral oscillators is dampened after a few cycles [31] once removed from the influence of the SCN. Furthermore, the expression pattern of the peripheral oscillators is delayed by several hours compared to the CCO [32]. For the pancreatic island, the self-sustained rhythm seems to last for several days in an ex vivo situation without dampening of the amplitude [17]. Future work will be aimed at elucidating islet-specific entraining cues and their role for regulating islet-linked functions such as insulin and glucagon release.

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