Developmental and Diurnal Dynamics of *Pax4* Expression in the Mammalian Pineal Gland: Nocturnal Down-Regulation Is Mediated by Adrenergic-Cyclic Adenosine 3′,5′-Monophosphate Signaling

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Pax4 is a homeobox gene that is known to be involved in embryonic development of the endocrine pancreas. In this tissue, Pax4 counters the effects of the related protein, Pax6. *Pax6* is essential for development of the pineal gland. In this study we report that *Pax4* is strongly expressed in the pineal gland and retina of the rat. Pineal *Pax4* transcripts are low in the fetus and increase postnatally; *Pax6* exhibits an inverse pattern of expression, being more strongly expressed in the fetus. In the adult the abundance of *Pax4* mRNA exhibits a diurnal rhythm in the pineal gland with maximal levels occurring late during the light period. Sympathetic denervation of the pineal gland by superior cervical ganglionectomy prevents the nocturnal decrease in pineal *Pax4* mRNA. At night the pineal gland is adrenergically stimulated by release of norepinephrine from the sympathetic innervation; here, we found that treatment with adrenergic agonists suppresses pineal *Pax4* expression *in vivo* and *in vitro*. This suppression appears to be mediated by cAMP, a second messenger of norepinephrine in the pineal gland, based on the observation that treatment with a cAMP mimic reduces pineal *Pax4* mRNA levels. These findings suggest that the nocturnal decrease in pineal *Pax4* mRNA is controlled by the sympathetic neural pathway that controls pineal function acting via an adrenergic-cAMP mechanism. The daily changes in *Pax4* expression may influence gene expression in the pineal gland. **(*Endocrinology* 150: 803–811, 2009)**

The Pax gene family encodes transcription factors that play crucial roles in metazoan developmental patterning and cell differentiation (1). Among the nine mammalian members of the Pax family, the homeobox genes *Pax4* and *Pax6* are known to be involved in development of the endocrine pancreas. *Pax6* is also known to play an essential role in development of the photodetector-derived neuroendocrine structure, the pineal gland (2, 3); this role is in accord with the reported expression of *Pax6* in the pineal gland during early developmental stages (4).

In the mouse embryo, *Pax4* is expressed in pancreatic islets. Expression rapidly declines toward the end of gestation; *Pax4* mRNA is undetectable or detectable at low levels in mature rodent islets (5–9). *Pax4* is essential for development of β-cells and appears to direct pancreatic endocrine precursor cells toward the β-cell lineage (10); in contrast, *Pax6* is involved in development of pancreatic α-cells from endocrine precursors (11). At the molecular level, Pax4 has been reported to act as a transcriptional repressor (5, 12, 13), which acts by binding the same *cis*-acting promoter sequences recognized by Pax6, thereby suppressing the expression of genes that are otherwise activated by Pax6, *e.g.* insulin and glucagon (5, 14–16).

Although it is clear that *Pax6* is expressed in the pineal gland and it plays a critical role in developmental biology of this tissue (2–4), knowledge of *Pax4* expression in the pineal gland is not

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Abbreviations: *Aanat*, Arylalkylamine *N*-acetyltransferase; DBcAMP, dibutyryl cAMP; E, embryonic d; Fra2, Fos-related antigen 2; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; Icer, inducible cAMP early repressor; NE, norepinephrine; P, postnatal d; qRT-PCR, quantitative real-time RT-PCR; SCGx, superior cervical ganglionectomy; ZT, zeitgeber time.

available. In view of this, we were especially interested in the results of preliminary microarray experiments, which indicated that *Pax4* is expressed in the pineal gland (S. L. Coon, D. Carter, R. Baler, and D. C. Klein, unpublished observations). This finding when viewed in the context of the biology of *Pax6* and the Pax4/Pax6 relationship discussed previously prompted us to study the expression of both *Pax4* and *Pax6* in the pineal gland. The results of these studies reveal unexpected features of the developmental and daily patterns of expression; of special interest is the finding that the *Pax4* mRNA levels increase during the day, when the pineal gland is considered to be relatively inactive in terms of circadian gene expression in general and melatonin synthesis in particular.

Materials and Methods

Animals

For the developmental series presented in Figs. 2 and 3 and supplemental Fig. S2, which is published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org, Sprague Dawley rats were obtained from timed-pregnant mothers (Charles River, Sulzfeld, Germany); the animals were housed under a 12-h light, 12-h dark schedule and decapitated at zeitgeber time (ZT) 6.

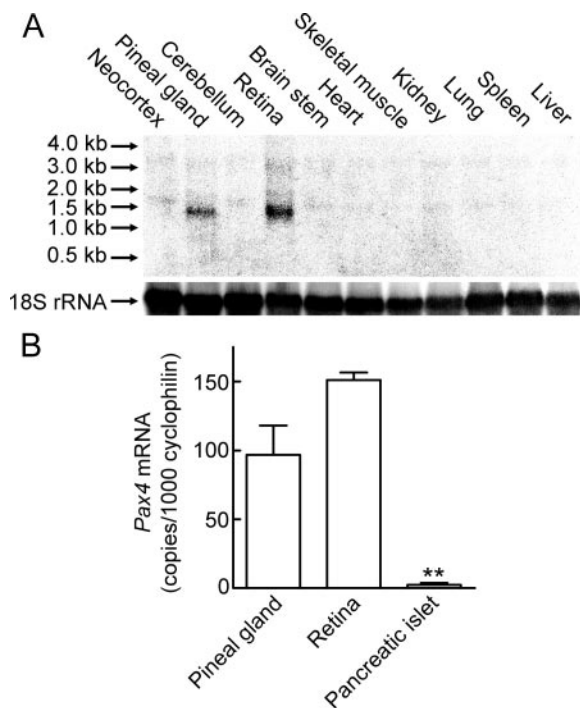

FIG. 1. Expression of *Pax4* in the pineal gland and retina of the adult rat. A, Northern blot analysis of *Pax4* expression in tissues removed from adult animals killed at ZT7. Arrows on the upper image indicate molecular weight markers. The *lower image* displays the same blot hybridized for detection of 18S rRNA. B, qRT-PCR analysis of *Pax4* expression in the pineal gland, retina, and pancreatic islet of adult animals killed at ZT7. PCR products were subcloned and sequenced confirming the expression of *Pax4* in all three tissues. Values on the *bar graph* represent the mean ± SEM of four to six independent RNA preparations. Statistical comparison of *Pax4* expression in the three tissues revealed a significant difference (one-way ANOVA, $F_{2,13}$ = 11.8; P = 0.0012); pair-wise comparison revealed significantly lower *Pax4* mRNA levels in pancreatic islets compared with the pineal gland (two-tailed Student's t test, t_5 = 4.4; P = 0.0071) and retina (two-tailed Student's t test, t_5 = 6.6; P = 0.0012). **, $P < 0.01$.

Brains and eyeballs were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and cryoprotected in 25% sucrose before freezing on crushed solid CO_2.

For the day-night radiochemical *in situ* hybridization experiments presented in Fig. 4, adult male Sprague Dawley rats (200–250 g; Charles River) were housed under a 12-h light, 12-h dark schedule. Bilateral superior cervical ganglionectomy (SCGx) was performed 10 d before euthanasia (17). Animals were decapitated at ZT6 and ZT18, and brains were removed immediately and frozen on crushed solid CO_2. For the *in vivo* experiment with isoproterenol treatment presented in Fig. 6, adult male Sprague Dawley rats (250–300 g) were housed under a 12-h light, 12-h dark schedule and injected (ip) with isoproterenol (Sigma-Aldrich, Steinheim, Germany; 10 mg/kg in PBS) or PBS at ZT5; animals were decapitated (ZT8), and brains were immediately removed and frozen on crushed solid CO_2.

For the *in vivo* quantitative real-time RT-PCR (qRT-PCR), Northern blot analyses, and RT-PCR analyses presented in Figs. 1 and 5 and supplemental Fig. S1, female Sprague Dawley rats (150–200 g; Taconic Farms, Germantown, NY) were housed under a 14-h light, 10-h dark schedule and killed by decapitation throughout the 24-h period at time points indicated in the figures and figure legends. Tissues were immediately frozen on crushed solid CO_2 and stored at −80 C.

For isolation of pancreatic islets, the pancreas of adult male Sprague Dawley rats (200–250 g; Taconic Farms) was infused with cold Liberase RI Enzyme solution (20 mg/ml; Roche Diagnostics, Indianapolis, IN) and digested for 30 min at 37 C. Islets were purified by filtration (0.5 mm mesh) and centrifugation in Histopaque-1077 (Sigma-Aldrich Corp., St. Louis, MO); islets were microscopically identified, collected, frozen on crushed solid CO_2, and stored at −80 C. For the organ culture and pinealocyte cell culture experiments presented in Fig. 7 and supplemental Fig. S3, respectively, pineal glands were obtained from adult female Sprague Dawley rats (150–200 g; Taconic Farms).

All experiments with animals were performed in accordance with the guidelines of *EU Directive 86/609/EEC* (approved by the Danish Council for Animal Experiments), the *National Institutes of Health Guide for Care and Use of Laboratory Animals* (approved by the Governing Board of the National Research Council), and the *Canadian Council on Animal Care Guidelines* (approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta).

Organ culture

Pineal glands were cultured as previously described (18, 19). After 48 h incubation (37 C, 95% O_2, 5% CO_2), the glands were transferred to a top-loading tabletop incubator. The glands were incubated for 1 h under control conditions or in medium containing 30 μg/ml actinomycin D or 50 μg/ml puromycin; this was followed by incubation for 12 h under control conditions or in medium containing 1 μM norepinephrine (NE) or 500 μM dibutyryl cAMP (DBcAMP). After treatment, glands were placed on crushed solid CO_2 and stored at −80 C.

Adenoviral transduction of pinealocytes

The procedures for culturing of pinealocytes, adenoviral transduction of constructs encoding short hairpin RNAs or full-length transcripts of inducible cAMP early repressor (*Icer*) and Fos-related antigen 2 (*Fra2*), and qRT-PCR evaluation presented in the supplemental data, were performed as previously described (20, 21).

Radiochemical *in situ* hybridization

Cryostat sections (12 μm, adult; 14 μm, developmental series) were hybridized with 38-mer [^{35}S]deoxy-ATP-labeled DNA probes as previously described (17, 22). For hybridization, the following probes were used: 5′-TCCAATCAGATGATGCACAGGATGGGTG-GTGAGGCAGG-3′, antisense, position 1053-1016 on rat *Pax4* mRNA (NM_031799); 5′-CCTGCCTCACCACCCATCCTGTGCAT-CATCTGATTGGA-3′, sense control, position 1016-1053 on rat *Pax4* mRNA (NM_031799); 5′-GCATCCTTAGTTTATCATACATGC-CGTCTGCGCCCATC-3′, antisense, position 605-568 on rat *Pax6*

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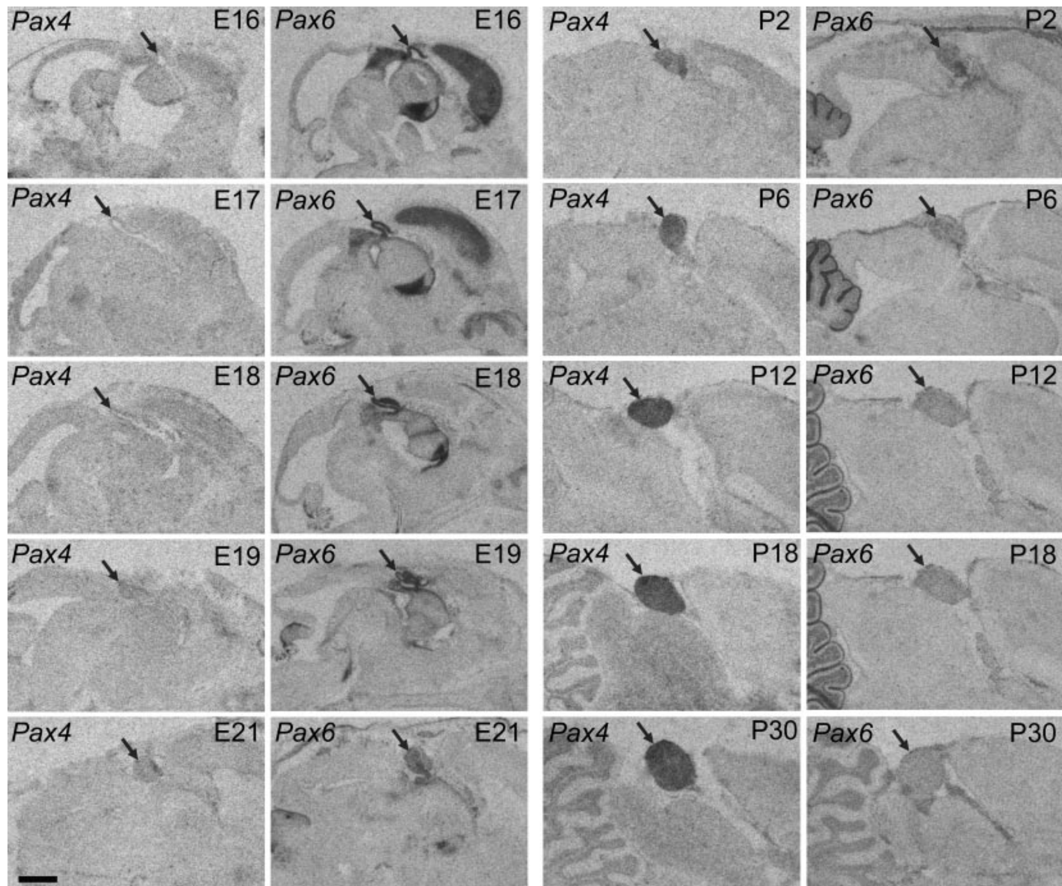

FIG. 2. Ontogenetic expression of *Pax4* and *Pax6* in the rat pineal gland. Autoradiographs of radiochemical *in situ* hybridization for detection of *Pax4* and *Pax6* mRNA in median sections of the brain in a developmental series ranging from E16 to P30. The pineal gland is marked by an *arrow*. Autoradiographs of sections hybridized with sense control probes are provided in supplemental Fig. S2. *Scale bar*, 1 mm.

mRNA (NM_013001.2); and 5′-GATGGGCGCAGACGGCATG-TATGATAAACTAAGGATGC-3′, sense control, position 568-605 on rat *Pax6* mRNA (NM_013001.2).

After hybridization and washing, the sections were exposed to an x-ray film for 2 wk. Images on the x-ray film were transferred to a computer and quantified (Image 1.42; Wayne Rasband, National Institutes of Health, Bethesda, MD). ODs from at least four random sections from each animal were converted to dpm/mg tissue using simultaneously exposed ^{14}C-standards calibrated by comparison with ^{35}S-tissue paste standards.

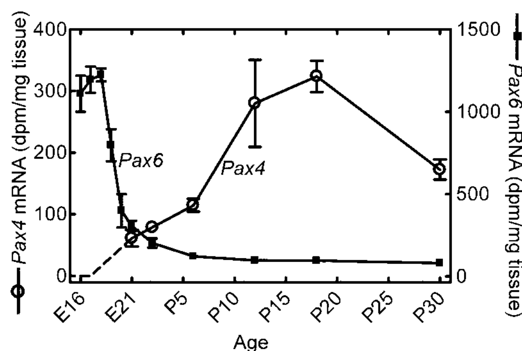

FIG. 3. Densitometric quantification of *in situ* hybridization autoradiographs of ontogenetic *Pax4* and *Pax6* gene expression in the pineal gland. In the earliest stages, a *Pax4* signal above background was not observed (*dotted line*). Values on graphs represent the mean ± SEM of three animals at each developmental stage examined. Differential expression levels during development were detected for *Pax4* (one-way ANOVA, $F_{5,12} = 11.4$; $P = 0.0003$); *Pax4* mRNA levels at P12 and P18 were significantly higher than that of earlier stages (Tukey's multiple comparison test, P values <0.05). Differential expression levels were also detected for *Pax6* (one-way ANOVA, $F_{10,22} = 59.2$; $P < 0.0001$); *Pax6* mRNA levels at E17 and E18 were significantly higher than those of other stages (Tukey's multiple comparison test, P values <0.05).

qRT-PCR

Total RNA was isolated using a RiboPure RNA isolation kit (Ambion, Inc., Austin, TX) or TRIZOL reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Total RNA was then subject to deoxyribonuclease treatment using TURBO DNA-free (Ambion) to remove contaminating genomic DNA. cDNA production was performed following the Superscript protocol (Invitrogen) using 1 μg deoxyribonuclease treated total RNA as starting material. Experiments were performed using a LightCycler 2.0 or a LightCycler 480 (Roche Diagnostics). Reactions (25 μl volume) contained 0.5 μM primers, RealTime SYBR Green master mix (SuperArray Bioscience, Frederick, MD) and cDNA according to the manufacturer's instructions. Primer sequences are given in Table 1. Assays included an initial denaturation step at 95 C for 10 min, proceeded by 40 cycles of a 95 C denaturation for 15 sec, 30 sec annealing at 63 C, then extension at 72 C for 30 sec. Product specificity was confirmed in initial experiments by agarose gel electrophoresis of the amplified products and thereafter during every qRT-PCR run by melting curve analysis (T_m). Less than 30 cycles were necessary to detect amplification. Transcript number was determined using internal standards; these were prepared by cloning the *Pax4*, *Pax6*, arylalkylamine *N*-acetyltransferase (*Aanat*), glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), and cyclophilin target PCR products into pGEM-T Easy vectors (Promega Corp., Madison, WI). Clone verification was performed by direct sequence analysis. For each experiment a set of 100-fold serial dilutions of each internal standard (10^1–10^7 copies/1 μl)

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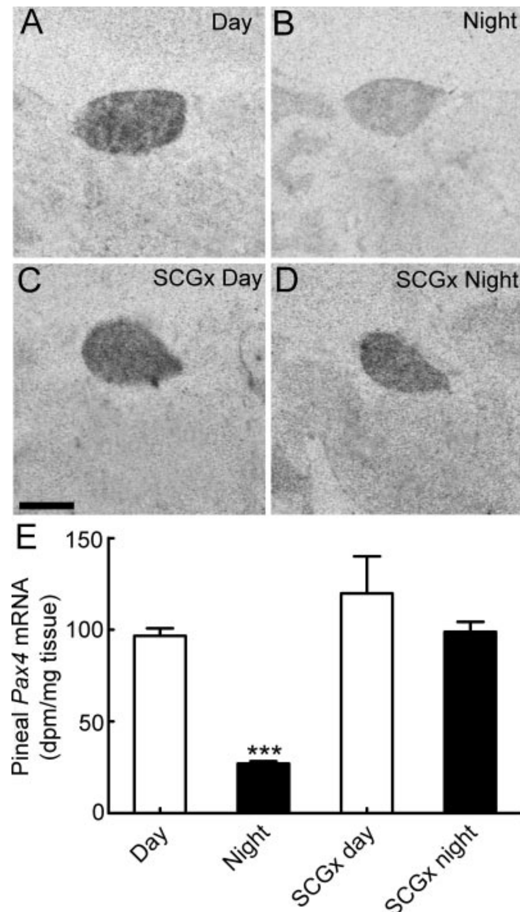

FIG. 4. Quantitative radiochemical *in situ* hybridization analysis of a diurnal variation in expression of *Pax4* in the pineal gland of the adult rat housed under a 12-h light, 12-h dark schedule. A, Autoradiograph of a median brain section from an animal killed at midday (ZT6). B, Autoradiograph of a median brain section from an animal killed at midnight (ZT18). C, Autoradiograph of a median brain section from a superior cervical ganglionectomized animal killed at midday (ZT6). D, Autoradiograph of a median brain section from a superior cervical ganglionectomized animal killed at midnight (ZT18). E, Densitometric quantification of *Pax4* mRNA in the rat pineal gland. Values on the *bar graph* represent the mean ± SEM of four to five animals in each experimental group. Two-way ANOVA analysis identified a significant effect of time of sampling ($F_{1,14}$ = 23.0; $P = 0.0003$) as well as SCGx ($F_{1,14}$ = 25.3; $P = 0.0002$). Pair-wise statistical comparison identified a significant day-night difference in pineal *Pax4* transcript levels in intact rats (two-tailed Student's *t* test, $t_5 = 16.4$; $P < 0.0001$); differences in pineal *Pax4* transcript levels among intact daytime rats and SCGx rats were not detected (one-way ANOVA, $F_{2,10}$ = 1.2; $P = 0.33$). ***, $P < 0.001$. *Scale bar*, 1 mm.

was prepared and used to generate standard curves. Transcript number was determined using a 2-μl sample of a 10-fold dilution of cDNA prepared as mentioned previously.

Northern blot analysis

Total RNA was prepared from frozen tissues using TRIZOL. A total of 8 μg total RNA was loaded per lane in a 1% agarose/0.7 M formaldehyde gel and separated by electrophoresis in a 1 × 3[*N*-morholino]propanesulfonic acid (50 mM) buffer (Quality Biological, Gaithersburg, MD). Membrane transfer was performed in 20× saline-sodium citrate transfer buffer by use of the Turboblotter system (Schleicher & Schuell, Keene, NH). Probe sequences were cloned into pGEM-T Easy vectors, and clone verification was performed by direct sequence analysis. DNA probes were generated by PCR (primer sequences are given in Table 1) using the sequenced plasmids as template and labeled with ^{32}P by random priming (Amersham Biosciences Inc., Piscataway, NJ). Hybridization, imaging, and stripping of blots were performed as previously described (22, 23). Transcript sizes were determined by comparison with standard RNA markers (Invitrogen).

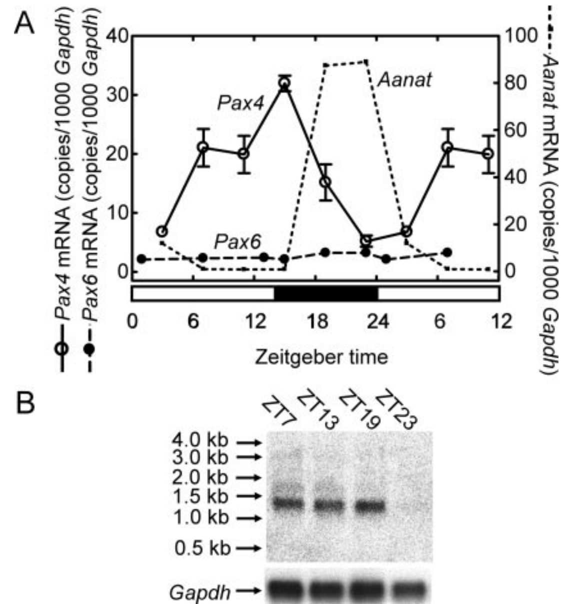

FIG. 5. Circadian expression of *Pax4* in the pineal gland of the adult rat. A, qRT-PCR analysis of diurnal expression of *Pax4*, *Aanat*, and *Pax6* in the pineal gland of the adult rat housed under a 14-h light, 10-h dark schedule. Nine animals were killed at each of six time points throughout the 24-h period. Values on *graphs* represent the mean ± SEM of three different pools of three glands at each time point. Significant changes in mRNA levels during the 24-h period were detected for both *Pax4* (one-way ANOVA, $F_{5,12}$ = 18.5; $P < 0.0001$) and *Aanat* (one-way ANOVA, $F_{5,12}$ = 17.8; $P < 0.0001$), but not in the case of *Pax6* (one-way ANOVA, $F_{5,12}$ = 2.4; $P = 0.097$). B, Northern blot analysis of *Pax4* expression in the pineal gland of adult rats housed under a 14-h light, 10-h dark schedule. Four to five animals were killed at each time point (ZT7, ZT13, ZT19, and ZT23). *Arrows on the upper image* indicate molecular weight markers. The *lower image* displays the same blot hybridized for detection of *Gapdh* mRNA.

RT-PCR analysis

RT-PCR analysis for differentiation of *Pax4* mRNA isoforms was performed by modification of a published method (24). Herculase II Fusion DNA Polymerase (Stratagene, La Jolla, CA) was used for PCR according to the manufacturer's recommendations; pineal and retinal cDNA was prepared as described previously, and primer sequences are given in supplemental Table S1, which is published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org. The PCR included 32 cycles of denaturation at 98 C for 30 sec, annealing at 62 C for 30 sec, and extension at 72 C for 45 sec. PCR products were subjected to electrophoresis in an ethidium bromide-stained 1.5% agarose gel. Product sizes were determined by comparison with a standard DNA ladder (Invitrogen).

Statistical analysis

A two-tailed Student's *t* test, one-way ANOVA, two-way ANOVA, or Tukey's multiple comparison test was used for comparing means of *in situ* hybridization signals (dpm/mg tissues) or the copy numbers obtained by qRT-PCR. A *P* value of less than 0.05 was considered to represent a statistically significant difference; for each analysis the obtained level of significance is indicated.

Results

Pax4 is highly expressed in the pineal gland and retina

Examination of *Pax4* expression in the adult rat by Northern blot revealed the presence of a tissue-specific band corresponding

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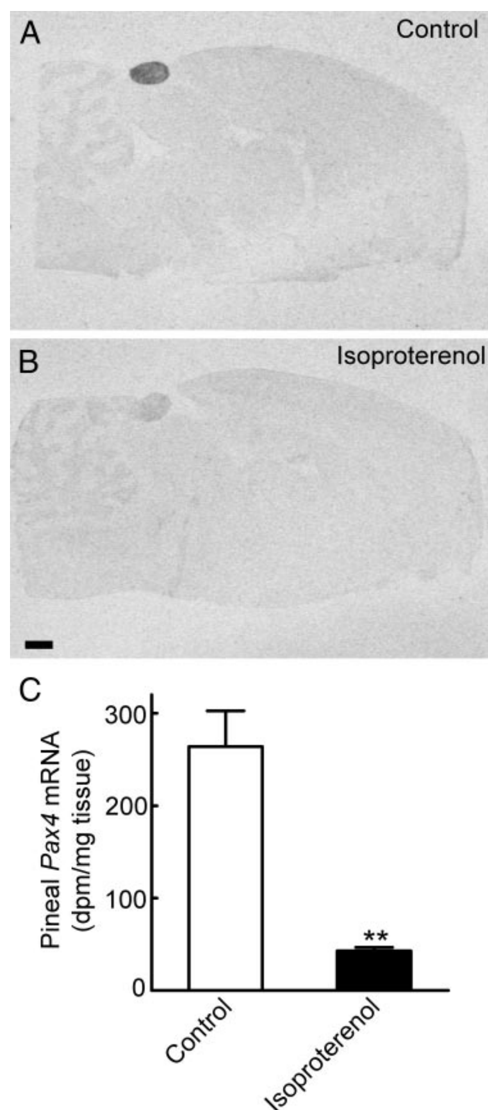

FIG. 6. Quantitative radiochemical *in situ* hybridization analysis of the effect of ip isoproterenol injection on the expression of *Pax4* in the pineal gland of the adult rat. A, Autoradiograph of a median brain section from a rat injected with PBS at ZT5 and killed at ZT8. B, Autoradiograph of a median brain section from a rat injected with isoproterenol (10 mg/kg) at ZT5 and killed at ZT8. C, Densitometric quantification of *Pax4* mRNA in the rat pineal gland. Values on the *bar graph* represent the mean ± SEM of three to five animals in each experimental group. A significant difference between animals injected with isoproterenol and control animals injected with PBS was identified (two-tailed Student's *t* test, $t_4 = 5.7$; $P = 0.0047$). **, $P < 0.01$. *Scale bar*, 1 mm.

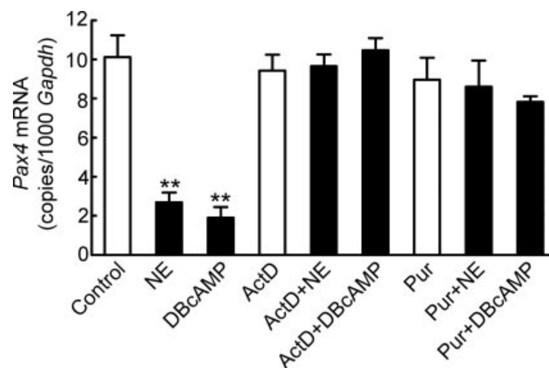

FIG. 7. qRT-PCR analysis of the effect of NE (1 μM) and DBcAMP (500 μM) in combination with actinomycin D (ActD) (30 μg/ml) and puromycin (Pur) (50 μg/ml) on the expression of *Pax4* in cultured pineal glands. Values on the *bar graph* represent the mean ± SEM of three different pools of glands in each experimental group. Two-way ANOVA analysis identified a significant effect of activation of the adrenergic system, *e.g.* NE and DBcAMP ($F_{2,18} = 15.8$; $P = 0.0001$), as well as blocking of gene product synthesis, *e.g.* actinomycin D and puromycin ($F_{2,18} = 47.8$; $P < 0.0001$); pair-wise comparison identified significant differences between the untreated control and the NE-treated glands (two-tailed Student's *t* test, $t_4 = 6.1$; $P = 0.0036$), and between the untreated control and the DBcAMP treated glands (two-tailed Student's *t* test $t_4 = 6.7$; $P = 0.0026$). Differences in levels of *Pax4* mRNA were not detected among control glands and glands treated with actinomycin D or puromycin (one-way ANOVA, $F_{6,14} = 1.0$; $P = 0.46$). **, $P < 0.01$.

to a transcript of approximately 1.3 kb in the pineal gland and retina (Fig. 1A); this transcript size corresponds to the full-length rat *Pax4* mRNA referred to as *Pax4a* (24). PCR analysis revealed the presence of both *Pax4a* and the shorter *Pax4*c transcript in the pineal gland, whereas the *Pax4b* and *Pax4d* isoforms were not detected (supplemental Fig. S1). Northern blot analysis was done with a probe that should theoretically detect all four isoforms; this did not reveal any truncated isoforms indicating that *Pax4a* is the major isoform expressed in this tissue.

As indicated previously, *Pax4* is expressed in the pancreatic islets of the adult rat (7, 8, 24). Here, we found that the abundance of *Pax4* mRNA in pancreatic islets was more than 20-fold lower than that in the pineal gland ($P < 0.01$, two-tailed Student's *t* test) and retina ($P < 0.01$, two-tailed Student's *t* test; Fig. 1B).

Expression of *Pax4* in the developing pineal gland

The ontogenetic expression of *Pax4* in the pineal gland was investigated using radiochemical *in situ* hybridization; sagittal brain sections of animals ranging from embryonic d (E) 16 to postnatal d (P) 30 were studied (Fig. 2).

At E16 the pineal gland is identified as a dorsal evagination of the most caudal part of the diencephalic roof; however, at this stage a *Pax4* hybridization signal was not present in the pineal gland or in any other areas of the brain. A signal was first detected at E21 in the epithalamic area, and prominent pineal expression of *Pax4* was detected thereafter. The signal was seen in all parts of the gland except in small areas likely to be perivascular spaces.

Densitometric quantification of *Pax4* mRNA in the pineal gland revealed an expression pattern changing during development ($P < 0.001$, one-way ANOVA); a low transcript level at E21 was followed by a peak during the second and third postnatal week ($P < 0.05$, Tukey's test; Fig. 3), and an intermediate level at P30.

Expression of *Pax6* in the developing pineal gland

Pax6 is known to be expressed in the mouse pineal gland (4). Here, we compared the developmental expression patterns of *Pax6* to those of *Pax4* in the pineal gland of the rat. A prominent signal was seen at E16 in the pineal recess, pretectal area, ventral thalamus, and neocortex; this pattern persisted during prenatal stages (Fig. 2). From E20, *Pax6* transcripts were detected in the cerebellum. During late embryonic and postnatal development, the thalamic and neocortical signal declined and were absent in the adult; however, *Pax6* mRNA was still detected in the pineal

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TABLE 1. Primer sequences for qRT-PCR and Northern blot analyses

Transcript	Gene accession no.	Position	Forward primer 5′-3′	Reverse primer 5′-3′	Analytical technique
Pax4	NM_031799	913–984	AGATGTTCCAGTGACACCACA	CACAGGAAGGAGGGAGTGG	qRT-PCR
Aanat	U38306	525–682	TGCTGTGGCGATACCTTCACCA	CAGCTCAGTGAAGGTGAGAGAT	qRT-PCR
Pax6	U69644	217–308	AACAGCGACGAAAGAGAGGA	CACTCTTTGAATAGAAGATCTCACACA	qRT-PCR
Cyclophilin	BC091153	359–426	TCTGCACTGCCAAGACTGAG	CATGCCTTCTTTCACCTTCC	qRT-PCR
Gapdh	BC059110	78–387	TGGTGAAGGTCGGTGTGAACGGAT	TCCATGGTGGTGAAGACGCCAGTA	qRT-PCR/Northern blot
Pax4	NM_031799	1–436	CTTGCCAGTTGGCTTTCTGT	CCCGAAGGACTCGATTGATA	Northern blot
18S rRNA	X01117	474–1070	CAGCAGGCGCGCAAATTACCCAC	ACGGTATCTGATCGTCTTCGAACC	Northern blot

gland and in the granular layer of the cerebellum, but at a low level.

Densitometric quantification of *Pax6* expression in the developing pineal gland revealed a changing temporal expression pattern ($P < 0.001$, one-way ANOVA; Fig 3). Maximal levels of *Pax6* mRNA were detected in the prenatal pineal at E17 and E18 ($P < 0.05$, Tukey's test); this was followed by a rapid decline just before birth. In the postnatal animal, low but sustained *Pax6* expression was detected. Accordingly, the expression pattern of *Pax6* in pineal gland exhibits an inverse temporal relationship to that of *Pax4*.

Diurnal expression of *Pax4* in the adult pineal gland

The known day/night rhythmic nature of the expression of many genes in the pineal gland prompted us to investigate if *Pax4* expression exhibits a 24-h rhythm in this tissue. *In situ* hybridization revealed a highly specific diurnal expression of *Pax4* in the pineal gland with highest levels of mRNA during the daytime (Fig. 4, A and B). Densitometric quantification of pineal *Pax4* mRNA revealed a significant day-night difference ($P < 0.001$, two-tailed Student's *t* test; Fig. 4E) with a day to night ratio of 3.6 ± 0.2.

The daily expression pattern of *Pax4* in the pineal gland was examined in detail using qRT-PCR; this revealed significant diurnal differences in *Pax4* mRNA levels (one-way ANOVA, $P < 0.001$; Fig. 5A). An increase in *Pax4* mRNA levels occurred during the light period, whereas expression declined gradually during the dark period. For comparison we measured the levels of *Aanat* mRNA, the abundance of which increases at night in pineal gland more than 100-fold (25, 26). *Aanat* transcripts encode the enzyme that controls the daily rhythm in melatonin production (27). The results clearly indicate that diurnal expression patterns of *Pax4* and *Aanat* are out of phase (Fig. 5A). In contrast, daily changes in the abundance of pineal *Pax6* mRNA were not detected ($P > 0.05$, one-way ANOVA). Using Northern blotting, we confirmed that the daily rhythm in *Pax4* mRNA reflected daily changes in the approximate 1.3-kb *Pax4* transcript with low values late during the dark period (Fig. 5B).

Adrenergic regulation of *Pax4* in the pineal gland

Daily rhythms in mammalian pineal function, including the daily rhythm in melatonin synthesis, are known to be controlled neurally by a pathway that includes the superior cervical ganglia (28–30). To determine whether the 24-h rhythm in expression of pineal *Pax4* is controlled by this pathway, the superior cervical ganglia were removed (SCGx). This abolished the detected day-night rhythm in pineal *Pax4* expression (two-way ANOVA, $P < 0.001$; Fig. 4E); the pineal *Pax4* transcript levels did not differ from daytime levels in control animals (one-way ANOVA, $P > 0.05$; Fig. 4, C–E).

The sustained high levels of *Pax4* mRNA observed in denervated pineal glands suggest that the rhythm in pineal *Pax4* expression may be adrenergically regulated by the release of NE from sympathetic nerve terminals in the gland. To investigate this, rats were treated with the β-adrenergic agonist isoproterenol during the day. This significantly suppressed pineal *Pax4* mRNA within 3 h to 20% the levels in control animals ($P < 0.01$, two-tailed Student's *t* test; Fig. 6). Similarly, treatment of cultured pineal glands with NE produced a significant suppression of *Pax4* mRNA ($P < 0.01$, two-tailed Student's *t* test; Fig. 7). NE is known to elevate pineal cAMP (31), which appears to mediate many effects of NE on gene expression. Here, we found that treatment with the cAMP mimic DBcAMP also significantly reduced *Pax4* mRNA ($P < 0.01$, two-tailed Student's *t* test; Fig. 7).

To determine whether suppression of *Pax4* mRNA levels by NE and DBcAMP requires mRNA and protein synthesis, the effects of actinomycin D and puromycin were investigated. These drugs significantly influenced the response of the cultured pineal glands ($P < 0.001$, two-way ANOVA); the level of *Pax4* mRNA in glands treated with actinomycin D or puromycin and NE or DBcAMP in combination did not differ significantly from that of control glands ($P > 0.05$, one-way ANOVA; Fig. 7). The results indicate that both *de novo* mRNA and protein synthesis appear to be required for the adrenergic-cAMP suppression of pineal *Pax4* transcript levels.

The finding that *de novo* protein synthesis is required for the inhibitory effects of NE on *Pax4* expression pointed to the possibility that synthesis of an immediate early gene product was required for this to occur. Two candidates for the role of such a hypothetical inhibitory transcription factor are Icer and Fra2, which are both expressed at night in the pineal gland in response to adrenergic signaling (32–34). However, elevation or suppression of the transcript levels of *Icer* or *Fra2* by adenoviral transduction of pinealocytes (20, 21) had no effect on the NE-induced suppression of *Pax4* transcript levels (supplemental Fig. S3).

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Discussion

The finding of high expression of *Pax4* in the pineal gland parenchyma of the postnatal rat provides the first body of evidence that this gene plays a role in the biology of this tissue. The finding that *Pax4* is not strongly expressed in the fetal pineal gland argues against a role in development and cell fate determination, in contrast to *Pax6*. Rather, it would appear that *Pax4* is more likely to play a role in regulating gene expression in the mature pinealocyte. This view is supported by the observation that the developmental appearance of *Pax4* mRNA is coincident with that of genes required for core pineal functions (35–39).

The developmental pattern of *Pax4* expression in the pineal gland is in marked contrast to that in the pancreas, in which the gene is strongly expressed early in development and then declines toward the end of gestation (5, 6, 10). Accordingly, it appears that whereas *Pax4* has a developmental role in the pancreas in cell fate determination, this may not be the case in the pineal gland, where, as discussed previously, it may have a regulatory role in the differentiated pinealocyte.

In the adult pineal gland, *Pax4* expression exhibits a marked diurnal rhythm with high transcript levels during the daytime. This suggests to us that it may play a role in modulating circadian gene expression. A striking feature of the rhythmic expression of *Pax4* in the pineal gland is that it is unlike that of the majority of cycling genes in the pineal gland, which exhibit a zenith during the night and a nadir during the day. In view of the evidence that Pax4 is known to act as a transcriptional repressor (5), it is possible that changes in *Pax4* mRNA are translated immediately into changes in Pax4 protein and that it plays a suppressive role in controlling daily changes in gene expression in the mature pineal gland. It is also possible that Pax4 protein exhibits slower changes that integrate preceding patterns of *Pax4* expression. However, efforts to address this are currently frustrated by the unavailability of useful reagents to detect Pax4 protein.

The marked difference in the expression patterns of *Pax4* and *Pax6* strengthens the view that these transcription factors have different roles. A developmental role for *Pax6* is indicated by the developmental pattern of expression described here, and by the results of studies indicating that *Pax6* maintains multipotency of retinal progenitor cells (40) and that the pineal gland fails to develop in *Pax6*-deficient animals (2). As indicated in the introductory section, Pax4 is known to suppress Pax6 *trans*-activating functions in pancreatic gene expression (5, 12–15). Because *Pax4* was also found to be expressed in the retina of the adult, we speculate that Pax4 might counteract the actions of Pax6 in the postnatal retina and pineal gland to ensure maintenance of the fully differentiated phenotype. However, this does not exclude the possibility that Pax4 acts independently of Pax6 to modify gene expression. Unlike *Pax4*, the nonrhythmic nature of *Pax6* leaves little reason to suspect that *Pax6* is directly involved in regulation of circadian biology.

The transcriptomes of the retinal photoreceptor cell and the pinealocyte are similar in that they include transcripts dedicated to photodetection and melatonin synthesis. This reflects a common evolutionary origin of these cell types from a common ancestral photodetector (41, 42). Both tissues express the same homeobox genes, including *Otx2* and *Crx* (22, 43–45). Although these are thought to have a primary role in regulation of developmental processes, *Otx2* and *Crx* are also expressed in the adult (22, 43), suggesting that they have a second role in maintaining phenotype. In this light the *Pax4* findings provide additional support for the view that homeobox genes in general may not be exclusively dedicated to developmental events and may be essential for normal function in the mature cell.

The rhythmic expression of *Pax4* in the pineal gland appears to be regulated by the sympathetic nervous system because removal of the superior cervical ganglia eliminated the rhythm in the pineal gland. Circadian information is transmitted from the endogenous circadian clock of the suprachiasmatic nucleus to the pineal gland via a multisynaptic neural pathway projecting through the superior cervical ganglia (29, 30); the daily rhythm in pineal *Pax4* mRNA is likely to be driven by the output of the suprachiasmatic nucleus. Daily oscillations in *Aanat* transcript levels and enzyme activity are known to be controlled by nocturnal release of NE from sympathetic nerve endings in the gland acting through an adrenergic/cAMP system (26, 27, 46–48). The findings of this report are consistent with the conclusion that the day-night rhythm in pineal *Pax4* mRNA is also controlled by an adrenergic/cAMP mechanism.

The evidence that the inverse diurnal expression of *Pax4* in the pineal gland is driven by down-regulation in response to nocturnal adrenergic stimulation is in marked contrast to numerous previous reports showing that the expression of several genes in the pineal gland peaks in the middle of the night with nocturnal expression being activated by adrenergic stimulation of the gland (19, 26, 32, 33, 49–52). Adrenergic repression of pineal gene expression has only been reported in the case of heparan sulfate 3-O-sulfotransferase 2 (53); the intracellular controlling mechanisms involved have not been resolved.

Our finding here that pineal *Pax4* transcript levels are not decreased after inhibition of transcription suggests that a posttranscriptional regulatory mechanism is involved in the dynamic oscillations in pineal *Pax4* expression. However, because *de novo* synthesis of gene products is essential for the suppressing effect of adrenergic stimulation, transcriptional induction of one or more factors seems to be involved in regulation of *Pax4* mRNA levels, perhaps by inducing nocturnal degradation of the transcript. Notably, posttranscriptional regulation of pineal *Aanat* mRNA may involve daily oscillations in RNA-destabilizing ribonucleoproteins (54).

The advances presented in this report provide reason to pursue the biology of *Pax4* in the pineal gland, including the molecular mechanisms involved in regulation of developmental and diurnal expression, and the role *Pax4* plays in controlling expression of other genes in this tissue.

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