Circadian Rhythm of Patched1 Transcription in the Pineal Regulated by Adrenergic Stimulation and cAMP*

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The tumor suppressor patched1 (PTC1), a product of the mammalian homologue of the Drosophila segment polarity gene patched, is a receptor for hedgehog (HH) and is crucial for embryonic development. Although little is known about the signal transduction pathways leading to the activation of ptc1, increased ptc1 transcription has always been associated with elevated HH activity and decreased activity of cAMP-dependent protein kinase A. Here, we demonstrate that in the mammalian pineal gland, ptc1 expression exhibits a dramatic diurnal rhythm with peak expression at midnight. ptc1 mRNA expression in the pineal is regulated by a clock mechanism mediated by the superior cervical ganglion. Most importantly, ptc1 transcription can be induced by agents activating the cAMP signal transduction pathway both in vivo and in vitro and appears to be independent of HH signaling.

Drosophila patched (ptc)† is a segment polarity gene (1) required for the correct patterning of larval segments during fly early development. It encodes a protein with 12 predicted transmembrane domains (2, 3) and functions to antagonize the action of HH signaling (4). Ptc is thought to antagonize HH signaling consequent to a physical interaction of Ptc with HH protein (5, 6). Mutations in human PTC1, the mammalian homologue of Drosophila Ptc, occur in the nevoid basal cell carcinoma syndrome, an autosomal dominant disorder characterized by predisposition to cancer, and in sporadic basal cell carcinoma, leading to the proposal that ptc1 is a tumor suppressor gene (7, 8).

One of the most conserved features of HH signaling is the transcriptional induction of ptc mRNA in both Drosophila and higher vertebrates including mammals. ptc expression is always associated with elevated HH activity. In the absence of HH, ptc expression is eliminated in late fly embryos, in fly wing imaginal discs, and in mammalian chondrocytes (9–11). Ectopic or abnormal expression of HH can induce ptc expression in adjacent cells in fly imaginal discs, in the developing neural tube and limb of the chicken, in the developing brain of the zebrafish, and in developing mouse embryos (12–16). In addition to HH proteins, cAMP-dependent protein kinase (PKA) regulates ptc expression. In most cases studied, increased PKA activity down-regulates HH target genes including ptc (17–25), suggesting that PKA is a universal negative regulator of HH signaling. Recently, however, a constitutively active form of catalytic subunit of mouse PKA, when introduced into fly embryos, was shown to elicit an up-regulation of ptc expression that appears to be independent of HH (26). This experiment, however, does not necessarily reflect the situation in vivo, leaving some doubt about whether PKA-mediated, HH-independent ptc induction exists under normal physiological conditions.

The pineal gland (epiphysis) is an unpaired midline neuroendocrine structure originating as an invagination of the diencephalon. The pineal transduces environmental light and dark information into nightly formation of the hormone melatonin, which links the body’s physiological processes to the daily cycle of sunlight and darkness. This circadian production of melatonin is dependent on the suprachiasmatic nucleus clock, information from which is relayed to the pineal by the superior cervical ganglion (SCG) in the form of rhythmic norepinephrine release at night. Light potently inhibits hormone production at night (27).

Subtractive hybridization cloning, seeking genes that are selectively up-regulated during the day or night, led to the identification of serotonin N-acetyltransferase (NAT) (28), the rate-limiting enzyme in melatonin synthesis, and a pineal night-specific ATPase (PINA) (29). Here we report the circadian expression of ptc1 in the pineal and its cAMP-dependent transcriptional activation that appears to be independent of the hedgehog signaling.

EXPERIMENTAL PROCEDURES

Animals—Harlan Sprague Dawley rats were purchased from Charles River Laboratories and housed in 14:10 light/dark lighting conditions with “lights off” at 9 p.m. for more than 1 week before the experiments. Harlan Sprague Dawley rats in which the superior cervical ganglia were bilaterally removed by surgery (superior cervical gangliectomized) were purchased from Zivic-Miller Laboratories (Allison Park, PA). During dark periods, animals were sacrificed under safe red lights (cut-off, 600 nm) by decapitation.

Subtractive Hybridization and Northern Blot Analysis of Pineal RNAs—The subtractive hybridization used to obtain a partial Ptc1 cDNA (clone NP008) has been previously described (28). The night-enriched clone NP008 encoding rat ptc1 corresponds to the 3'-untranslated region (nucleotide 4386–4805, GenBank™ accession no. U43148) of human ptc1. NP008 was then used to screen a rat pineal night cDNA library to obtain a number of overlapping rat ptc1 cDNAs, all of which show sequence homology of more than 92% with the published mouse ptc1 sequence. The longest clone (NP008.8), which was completely

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1 The abbreviations used are: ptc1, patched1; HH, hedgehog; PKA, cAMP-dependent protein kinase; SCG, superior cervical ganglion; NAT, serotonin N-acetyltransferase; PINA, pineal night-specific ATPase.

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sequenced on both strands, was used for Northern blot analysis.

In Situ Hybridization Analysis—ptc1 (3′-untranslated sequence of the NP008.8) and the full-length rat NAT cDNA (28) probes were used for in situ hybridization studies. The in situ hybridization technique was performed as previously described (30).

Pineal Organ Cultures—Pineal glands were cultured in vitro according to a modified procedure (31) of Parfitt et al. (32). Briefly, freshly exercised glands were cleaned of adhering tissue and cultured in BGJb medium (Fitten-Jackson Modification, Life Technologies, Inc.) containing 1 mg/ml BSA. Immediately before use, sodium ascorbate and glutamine were added to final concentrations of 0.5 and 2 mM, respectively. Cultures were grown in 24-well culture plate containing 0.2 ml of medium with the medium changed every 24 h. Each well contained two pineals supported on the middle of a circular nylon mesh.

RESULTS

Ptc1 Is Diurnally Expressed in the Pineal—In a second round of screening the subtracted night-specific pineal cDNA library, we found that ptc1 expression in the pineal is night-specific with very little expression of ptc1 during the day (Fig. 1). Peak nighttime levels are more than 15-fold greater than daytime troughs. Significant elevation of ptc1 expression, first evident at 10 p.m. (2200 h), rises rapidly to peak levels at 12 p.m. (2400 h), which are maintained until a precipitous decline at 8 a.m. This rhythm differs from that of NAT and PINA in that ptc1 mRNA reaches its peak earlier (Fig. 1B and data not shown).

Ptc1 Expression Is Regulated by a Central Clock via the SCG—Temporal expression of ptc1 was examined in rats maintained in constant darkness. The diurnal rhythm of ptc1 expression is maintained in the absence of light, indicating that it is under the control of a biological clock (Fig. 2A, left panel). Constant light exposure, which is the most potent rhythm suppressor of clock-regulated pineal genes, abolishes the ptc1 rhythm in the rat pineal (Fig. 2A, right panel), demonstrating that typical environmental modulators of the circadian rhythm can disrupt ptc1 expression. Bilateral ablation of the SCG abolishes ptc1 transcription (Fig. 2B), consistent with the notion that ptc1 circadian gene expression requires neuronal input from the SCG.

Ptc1 Induction in the Pineal Appears To Be Independent of Hedgehog Signaling—Hedgehog is the most potent activator of the PTC transcription. We therefore searched for expression of all known mammalian hedgehog (hh) genes in the adult pineal gland. No expression of any of the three mammalian hh homologues (33) is detected in the rat pineal gland by Northern blotting and in situ hybridization during the day or night. Because sympathetic innervation from the SCG is thought to be the sole innervation of the pineal, we also searched for the hh messages in the SCG by Northern and in situ analysis but failed to detect expression of hh transcripts at any time point.

Hedgehog proteins travel considerable distances from their sites. During eye development in Drosophila, for instance, HH moves alone retinal axons to the brain to induce lamina neurogenesis (34). For test to the possibility of HH protein expression in the pineal, we performed Western blot and immunohistochemical analysis using extracts and sections of day and night pineals utilizing the sonic HH antibody 5E1 (35) and failed to detect any signal. The absence of hh expression in the pineal gland and in the SCG suggests that it may not be a direct physiologic regulator of Ptc1 in the pineal.

Activation of cAMP Signaling Is Necessary for Induction of Ptc1 Transcription—We tested whether norepinephrine, the adrenergic neurotransmitter of the SCG that activates melanin synthesis, also activates ptc1 expression. In vivo, this appears to be the case, as administration of the β-adrenergic agonist isoproterenol to intact animals induces expression of
ptc1 in the pineal gland (Fig. 3A). To directly examine the regulation of ptc1 by the β-adrenergic system, we utilized the pineal gland in an organ culture (Fig. 3B) (29). Norepinephrine strongly stimulates ptc1 expression in organ culture. The stimulation is unaffected by α-adrenergic antagonist prazosin but is completely blocked by the β-adrenergic antagonist propranolol. Furthermore, isoproterenol stimulates ptc1 expression in pineal organ culture, as it does in vivo, whereas the α-adrenergic agonist phenylephrine is ineffective (Fig. 3A). Thus activation of β-adrenergic receptors is sufficient for ptc1 induction in the pineal gland. Because activation of β-adrenergic receptors leads to the activation of adenylyl cyclase with increases in intracellular cAMP, we examined the effect of direct cAMP stimulation on the induction of ptc1 mRNA. Forskolin and a non-hydrolyzable cAMP analogue, dibutyryl cAMP, both stimulate ptc1 expression (Fig. 3B). These results indicate that activation of the cAMP signal transduction pathway is necessary for the induction of ptc1 transcription in the pineal.

**PtC1 Induction Is Regulated Differently from the Pineal-specific Night Transcripts**—The diurnal pattern of expression of ptc1 in the pineal led us to postulate that it may participate in modulation of melatonin production. As an initial study to determine how ptc1 may participate in this process, we tested the developmental expression profiles of ptc1 transcript in the pineals and compared it with the pineal-specific and night-specific transcript, serotonin NAT (Fig. 4; see Refs. 28 and 29). Although the circadian expression patterns of ptc1 are similar to that of NAT in adult pineals, ptc1 mRNA is not up-regulated in night pineals at postnatal day 2 (P2), unlike that of NAT. These experiments indicate that ptc1 expression during early pineal development is regulated differently from that of pineal-specific night transcript known to drive melatonin production. Further, it appears that circadian expression of ptc1 is not required for the rhythmic transcription of NAT.

**DISCUSSION**

Mechanisms of HH/PTC signal transduction have been the subject of intense investigation. In this study, we demonstrate that the ptc1 transcript displays a pronounced circadian rhythm in the pineal. In the pineal, cAMP activates ptc1 transcription, contrasting dramatically with ptc1 regulation in other systems. HH signaling does not seem to be required in pineal ptc1 induction, again contrasting with other ptc systems. These observations point to a novel pathway for ptc1 signal transduction that is driven by cAMP.

Is cAMP the only signaling system driving pineal ptc1 expression? Our results demonstrate that cAMP is required to induce ptc1 expression. Although it is possible that the HH system may be involved in pineal regulation, several factors support a predominant role of cAMP. First, we have repeatedly failed to detect any mRNA or protein expression for any of the HH family members in pineal. Second, there is no evidence of HH activity in SCG, the only known neuronal input to the pineal. Third, because the pineals in vitro in the organ culture experiments were analyzed more than 48 h after excision, nerve terminals had completely degenerated. The denervated pineals are able to mount an impressive increase in ptc1 expression by cAMP alone, without other factors (such as HH) that may be carried by SCG neurons in vivo. Fourth, in vivo

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**Fig. 3.** Stimulation of ptc1 expression by agents activating the cAMP signal transduction pathway *in vivo* and *in vitro*. A, Northern blot analysis of pineal RNAs from rats injected with β-adrenergic receptor agonist isoproterenol (+ISO) and vehicle (−ISO). Rats were given intraperitoneal injection of isoproterenol (1 ml of 100 μg/ml) at 6 p.m. and sacrificed at 9 p.m. Control rats were injected with the vehicle. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. B, Northern analysis of RNA isolated from pineals cultured *in vitro* in the presence of various drugs. Pineals were cultured in BGJb medium for 48 h to allow nerve terminal degeneration prior to drug stimulation. All of the drugs were used at 1 μM concentration, except norepinephrine (NE), which was used at 0.1 μM. The organ culture experiment was performed as described (31). PRAZ, prazosin; PROP, propranolol; FHE, phenylephrine; ISO, isoproterenol; FSK, forskolin; DB-cAMP, dibutyryl cAMP.

**Fig. 4.** Midsagittal brain sections of postnatal day 2 (P2) and adult rats during the day (4 p.m.) and night (4 a.m.) were processed for *in situ* hybridization with digoxigenin-labeled ptc1 (NP008.8) and NAT riboprobes. The daytime expressions of both Patched1 (A) and NAT (C) are clearly visible in sections from postnatal day 2 rats compared with those of the adult sections (E and G). Whereas NAT mRNA displayed marked diurnal in both P2 (C and D) and adult pineals (G and H), circadian rhythm of ptc1 transcription is not yet seen in P2 (A and B) animals although it is marked in adult pineals (E and F). Sense probes revealed no positive signals (not shown).
ablation experiments indicate that expression of ptc1 is dependent on SCG input but can be fully recovered using agents that specifically stimulate cAMP production.

The cAMP-dependent and HH-independent regulation of ptc1 in the pineal suggests PTC1 may play a role in the neuroendocrine and perhaps other systems in an activity-dependent manner. Unlike the relatively slow time course of ontogenic and oncogenic processes in which ptc1 has been previously studied, the pineal regulation of ptc occurs rapidly, with 10–20-fold changes of expression in a few hours. This swift change suggests a novel type of short term regulatory function for PTC1 in the pineal and other HH-independent PTC systems. Our developmental studies indicate that ptc1 cycling is not necessary for the rhythmic transcription of NAT early in life. Perhaps PTC1 in the pineal functions in the post-transcriptional regulation of diurnal processes (i.e. mRNA stability or post-translational modifications). Besides some diurnally determined role, PTC1 might play a part in the ontogenesis of pineal region tumors, consistent with the known functions of PTC1 during development and in tumor formation.

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REFERENCES
1. Nusslein-Volhard, C., and Wieschaus, E. (1980) Nature 287, 795–801
2. Nakano, I., Hidalgo, A., Taylor, A., Whittle, J. R., and Ingham, P. W. (1989) Nature 341, 508–513
3. Hoesper, J. E., and Scott, M. P. (1989) Cell 59, 751–765
4. Ingham, P. W. (1998) Curr. Opin. Genet. Dev. 8, 88–94
5. Stone, D. M., Hynes, M., Armanini, M., Swanson, T. A., Gu, Q., Johnson, R. L., Scott, M. P., Pennica, D., Goddard, A., Phillips, H., Noll, M., Hoesper, J. E., de Sauvage, F., and Rosenthal, A. (1996) Nature 384, 129–134
6. Marigo, V., Davey, R. A., Zuo, Y., Cunningham, J. M., and Tabin, C. J. (1996) Nature 384, 176–179
7. Hahn, H., Wicking, C., Zaphiropoulos, P. G., Gailani, M. R., Shanley, S., Chidambaram, A., Vorechovsky, I., Holmberg, E., Unden, A. B., Gillies, S., Negus, K., Smyth, I., Pressman, C., Leffell, D. J., Gerrard, B., Goldstein, A. M., Dean, M., Toftgard, R., Chenevix-Trench, G., Wainwright, B., and Bale, A. E. (1996) Cell 85, 841–851
8. Johnson, R. L., Rothman, A. L., Xie, J., Goodrich, L. V., Bare, J. W., Bonifas, J. M., Quinn, A. G., Myers, R. M., Cox, D. R., and Epstein, E. H., Jr., and Scott, M. P. (1996) Science 273, 1668–1671
9. Hidalgo, A., and Ingham, P. (1996) Development 120, 291–301
10. Capdevila, J., and Guerrero, I. (1994) EMBO J. 13, 4459–4468
11. Vortkamp, A., Lee, K., Lanske, B., Segre, G. V., Kronenberg, H. M., and Tabin, C. J. (1996) Science 273, 613–622
12. Tabata, T., and Kornberg, T. B. (1994) Cell 76, 89–102
13. Marigo, V., and Tabin, C. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9346–9351
14. Marigo, V., Scott, M. P., Johnson, R. L., Goodrich, L. V., and Tabin, C. J. (1996) Development 122, 1225–1233
15. Concordet, J. P., Lewis, K. E., Moore, J. W., Goodrich, L. V., Johnson, R. L., Scott, M. P., and Ingham, P. W. (1996) Development 122, 2835–2846
16. Goodrich, L. V., Johnson, R. L., Milenkovic, L., McMahon, J. A., and Scott, M. P. (1996) Genes Dev. 10, 301–312
17. Pan, D., and Rubin, G. M. (1995) Cell 80, 543–552
18. Jiang, J., and Struhl, G. (1995) Cell 80, 563–572
19. Li, W., Ohlmeyer, J. T., Lane, M. E., and Kalderon, D. (1995) Cell 80, 553–562
20. Lepage, T., Cohen, S. M., Diaz-Benjumea, F. J., and Parkhurst, S. M. (1995) Nature 373, 711–715
21. Strutt, D. I., Wiersdorff, V., and Mlodzik, M. (1995) Nature 373, 705–709
22. Fan, C. M., Porter, J. A., Chiang, C., Chang, D. T., Beachy, P. A., and Tessier-Lavigne, M (1995) Cell 81, 457–465
23. Hammerschmidt, M., Bitgood, M. J., and McMahon, A. P. (1996) Development 122, 2885–2894
24. Epstein, D. J., Marti, E., Scott, M. P., and McMahon, A. P. (1996) Development 122, 2835–2846
25. Concordet, J. P., Lewis, K. E., Moore, J. W., Goodrich, L. V., Johnson, R. L., Scott, M. P., and Ingham, P. W. (1996) Development 122, 2835–2846
26. Ohlmeyer, J. T., and Kalderon, D. (1996) Genes Dev. 11, 2250–2258
27. Borjigin, J., Li, X., and Snyder, S. H. (1999) Annu. Rev. Pharmacol. Toxicol. 39, 53–85
28. Borjigin, J., Wang, M. M., and Snyder, S. H. (1995) Nature 378, 783–785
29. Borjigin, J., Payne, A. S., Deng, J., Li, X., Wang, M. M., Ovodenko, B., Gitlin, J. D., and Snyder, S. H. (1999) J. Neurosci. 19, 1018–1026
30. Blackshaw, S., and Snyder, S. H. (1997) J. Neurosci. 17, 8074–8082
31. Yuzilier, A. (1983) J. Neurochem. 41, 146–153
32. Parfitt, A. G., Weller, J. L., and Klein, D. C. (1976) Neuropharmacology 15, 353–358
33. Eriksson, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A., and McMahon, A. P. (1993) Cell 75, 1417–1430
34. Huang, Z., and Kunes, S. (1996) Cell 86, 411–422
35. Ericson, J., Morton, S., Kawakami, A., Roehl, H., and Jessell, T. M. (1996) Cell 87, 661–673
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