- Rapid Communication -

Analyses of Serotonin N-Acetyltransferase mRNA Content in Rat Pineal Gland and Testis by an Improved Assay System- Micro Lysate Protection Assay

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Abstract: A quick and reliable method, micro lysate protection assay for quantitation of mRNA encoding serotonin N-acetyltransferase (AANAT) was developed. AANAT mRNA content in a single pineal gland could be measured by this method. In addition to the pineal gland, a day-night rhythm in AANAT mRNA content was also observed in the rat testis.

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A circadian rhythm in circulating melatonin content generated by the pineal gland regulates many circadian functions and seasonal reproduction^{1, 5)}. The rate limiting enzyme of melatonin synthesis is serotonin N-acetyltransferase (arylalkylamine N-acetyltransferase, AANAT, EC2.3.1.87) which converts serotonin to N-acetylserotonin^{2, 7)}. The activity of AANAT in the pineal gland is also low during daytime and high during nighttime thereby creates diurnal melatonin rhythm^{2, 7)}.

Molecular cloning of rat AANAT cDNA has recently been achieved and provided a means of determining AANAT mRNA levels^{4, 11)}. It has been reported that AANAT mRNA content in the pineal gland also fluctuates in a pattern similar to the enzyme activity, indicating that the change in AANAT activity is attributable to synthesis and degradation of its mRNA^{4, 11)}. As the rat pineal gland is so small as 1-2 mg, RNA preparations from pooled pineal glands have been used in Northern blot analysis of AANAT mRNA levels^{4, 11)}. Here we employed a lysate RNase protection assay⁶⁾ and modified it to be suitable for analysis of mRNA content in very small tissues. We report AANAT mRNA contents in a single rat pineal gland and testes in light and dark period measured by this system.

Materials and Methods

Complementary DNA corresponding to nucleotide 291-690 basepairs of rat AANAT cDNA (GenBank accession No.U40803) was obtained by reverse transcriptase-PCR and subcloned into a vector containing T7 RNA polymerase promoter (pCRII, invitrogen). An antisense RNA probe was synthesized as described previously¹⁰⁾.

Male Donryu rats were fed on a laboratory chaw under a 12L:12D cycle (lights on at 6:00). Pineal glands and testes were taken at 12:00 under ordinary fluorescent light or at 24:00 under dim red light.

Micro Lysate Protection Assay: Each pineal gland was transferred to a microhomogenizer (Radnoti) containing 50 μ l lysis buffer (5M guanidine thiocyanate, 0.1 M EDTA, 0.1 M Tris-HCl, pH 7.0, 1 % 2-mercaptoethanol) and homogenized. A small piece of testis was weighed and homogenized in a microhomogenizer containing 5 volumes of lysis buffer. Homogenates were transferred to microcentrifuge tubes and centrifuged at 15,000 rpm for 5 min at room temperature. Forty-five microliters of the supernatant were mixed with 5 μ l of lysis buffer containing 250,000 cpm of antisense RNA probe. After hybridization for overnight at 37°C, 500 μ l digestion solution (10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 300 mM NaCl, 80 mg/ml RNase A, 4 mg/ml RNase T1) was added and the tubes were incubated at 37°C for 30 min. Twenty microliters of 10 % sodium N-lauroyl-sarcosinate and 10 μ l of proteinase K solution (RNA grade, BRL) were added and the tubes were incubated at 37°C for 30 min. The tubes were centrifuged for 2 min at room temperature. The supernatant and 500 μ l isopropanol were mixed, kept at -20°C for 15 min, and then centrifuged for 15 min. The precipitates were dried and resuspended in sample buffer⁹). The protected probe was separated on denaturing polyacrylamide gel and visualized as described previously¹⁰).

Results and Discussion

As is shown in Fig.1, a discrete band of expected size was obtained from the lysate of a single pineal gland of the rat. This method possesses following advantages over conventional Northern blot analysis. First, only one pineal gland is required for one assay. Messenger RNA content per each organ or per wet weight is directly estimated, eliminating the necessity of measurement of control mRNA such as β -actin or G3PDH mRNA^{4, 11}).

Fig.1. Representative results of AANAT mRNA content in one of rat pineal gland measured by micro lysate protection assay. Rats were housed in a controlled lighting environment (LD12:12, lights on at 6:00).

Day and night time AANAT mRNA levels in rat pineal glands and testes are shown in Fig. 2. Pineal AANAT mRNA levels were more than 50 times higher in dark period than in light period (Fig.2, left), which agreed with previous reports^{4, 11}). The AANAT levels in testes were also elevated (3-fold) at night (Fig.2, right), indicating that diurnal rhythm of AANAT mRNA exists in the rat testis. Day/night rhythm of AANAT gene expression has been shown in pineal gland and retina^{4,11}). In the testis, however, no attention has been paid to diurnal AANAT mRNA rhythm. This observation is interesting since no diurnal rhythm existed in the testis of Japanese quail (Fu et al., unpublished data).

The mechanism and physiological significance of the rhythm of testicular AANAT mRNA is hard to speculate. In the pineal gland and retina, several factors are known to cause circadian

rhythm of AANAT gene expression, i.e. light detector, input from the suprachiasmatic nucleus of the hypothalamus and/or their own endogenous circadian oscillators⁸). In the case of the testis, no such factors are elucidated. The testes contained 2 times greater amount of AANAT mRNA than the pineal gland, when weights of these organs are taken into account. As the activity of hydroxyindole-*O*-methyltransferase (HIOMT) which converts N-acetylserotonin to melatonin is known to very low in extrapineal tissues³), the testis may not be an organ which actually produces melatonin. Even if this is the case, our result suggests that the testis has an unknown role in serotonin metabolism which is under the control of circadian rhythm.

Fig.2. Diurnal rhythm of AANAT mRNA in rat pineal gland (left) and testis (right). Rats were maintained under a controlled lighting condition (a 12 h-12 h LD cycle, lights on from 6:00), and sacrificed at 12:00 in the light period and 24:00 in the dark period. Open circles represent individual data and filled circles represent the mean of individual data at each time point (n=5).

In conclusion, we developed a modified lysate protection assay which enabled us to measure AANAT mRNA content of a single rat pineal gland. We have shown that a day-night rhythm in AANAT mRNA exists in the rat testis, in addition to the pineal gland.

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