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Expression of Cholecystokinin Type A Receptor Gene Correlates with DNA Demethylation during Postnatal Development of Rat Pancreas

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Cholecystokinin stimulates pancreatic amylase secretion, gallbladder contraction, and pancreatic growth, etc. by binding with high affinity to a cholecystokinin type A receptor (CCKAR). To better understand the expression of CCKAR mRNA in terms of tissue specificity and postnatal development, we determined the methylation status of *Bss*HII sites (5′-B sites) in the rat CCKAR gene promoter. The 5′-B sites in adult pancreas expressing CCKAR mRNA were much less extensively methylated than those in fetal pancreas not expressing the mRNA. In brain, liver, and kidney of adult rats not expressing CCKAR mRNA, the 5′-B sites were methylated. In pancreas, the demethylation level of the sites increased at 21 days after birth. Concomitant with the DNA demethylation level in the 5′-B sites, the mRNA level rose rapidly in 21 days. These results demonstrate that methylation and expression of the CCKAR gene reveal a good inverse correlation. © 1999 Academic Press

Cholecystokinin (CCK) is a peptide hormone that has numerous important physiologic functions, mediated through receptors. The receptors can be subdivided pharmacologically into cholecystokinin type A (CCKAR) and B receptors, by their binding affinities for CCK/gastrin family peptides (1). In the gastrointestinal system, CCKAR mediates the physiologic functions that result on binding of CCK, such as pancreatic zymogen secretion, gallbladder contraction, pancreatic growth and regulation of carbohydrate metabolism through augmentation of pancreatic islet insulin secretion (1). In addition, there is general agreement that CCK may also act as a promoter of pathologies such as pancreatic tumorigenesis (2) or pancreatitis (3, 4). We and others have previously reported that rat CCKAR is expressed in a tissue-specific and postnatal-dependent manner. In rats, CCKAR mRNA is found in the adult pancreas (5), fundus mucosa (6) and AR42J cells (7), and is not found in the fetal pancreas (5), adult liver, kidney, subcortex, cortex and muscle (7, 8) by Northern analysis. We also isolated rat genome clones containing the entire coding region of CCKAR and upstream regulatory elements, and determined the expected transcription start site (9). In expression of CCKAR mRNA, no attempt has yet been made to investigate the mechanism of the transcriptional regulation of expressions in a tissue-specific and postnatal-dependent manner.

DNA methylation at cytosine residues in the CpG dinucleotide is recognized as an important mechanism of epigenetic regulation of gene expression (10). Generally, there is inverse correlation between the methylation of cytosine and the gene expression. Therefore, we considered that methylation of the CCKAR promoter region may contribute significantly as the one of the transcriptional regulators in a tissue-specific and postnatal-development manner. In the present study, our results apparently demonstrate that the methylation of CpG-rich segments of the CCKAR promoter is correlated with less expression of CCKAR gene.

MATERIALS AND METHODS

Animals. Male and female Wistar rats were obtained from Clea Japan Inc. All rats were freely given a standard diet (CRF-1; Oriental Yeast, Tokyo, Japan). Fetuses rats were obtained from 8- to 9-week-old female rats on day 19 of gestation. Parturition of rats occurred on day 21 of gestation, which was regarded as the day of birth (day 1).

Cell culture. The AR42J and ARIP cell lines were obtained from Dainippon Pharmaceutical Co., Japan. The cells were maintained with Digo's T medium (Nihon Seiyaku Co., Japan) supplemented

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with 10% fetal calf serum (GIBCO BRL Co., U.S.A.) at 37°C under a humidified atmosphere of 5% CO_2 in air.

Probe preparation. To obtain probe (415 bp) for Southern and Northern blot analysis, exon 1 and 2 of the CCKAR cDNA (7) was amplified by PCR from a plasmid bearing the cDNA of the rat CCKAR (kindly provided by Dr. S. A. Wank, NIH, Bethesda, MD) using the sense primer 5′-AGGAGAGAGACAGGAATGAGC-3′ and antisense primer 5′-AGGTGGTAGTCTTGC-ACACG-3′ and *Taq* polymerase (Promega). The conditions for PCR were 30 cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C.

Southern and Northern blot analysis. Southern and Northern blot analysis was performed as described by previous reports (5, 9). In developmental studies, tissues were sampled on day 3, 7, 14, 21, and 35 after birth. For genomic DNA and total RNA from each tissue, three to ten rats at the same age of development were sampled. In Southern and Northern analysis, two independent experiments were performed on the genomic DNA and total RNA from each newborn rats but significant difference was not observed in these results. Autoradiograms were analyzed with a computer scanner and NIH image program.

RESULTS

To determine the profile of DNA methylation in the CG-rich segment (Fig. 1A) of the CCKAR gene, we employed Southern blot analysis using the methyl-sensitive restriction enzyme, *Bss*HII. The CG-rich segment contains four *Bss*HII sites (5′-B sites, bold "B" in Fig. 1A) at intervals of 2 bp and is found 114 to 132 bp upstream from the expected start site for CCKAR transcription (9). In Southern blot analysis, these sites were not separable by electrophoresis on 1% agarose.

Previously, we have reported that rat CCKAR mRNA is expressed in adult but not in fetal pancreas by Northern analysis (5). To compare the status of DNA methylation of 5′-B sites in the pancreas, genomic DNA was extracted from adult and fetal (as littermates) rat pancreas and digested with *Sac*I (as a positive control for Southern blot), *Bss*HII, *Sac*I + *Bss*HII and *Xba*I + *Bss*HII (Fig. 1B). Digestion of the genomic DNA with *Bss*HII revealed an intact 2.6-kb DNA fragment in adult, but no band in the fetus (in range from 1.9 to 8.4 kb). When pancreas DNA was digested with *Sac*I + *Bss*HII, 1.9 and 4.9 kb bands were found both in adult and fetus. The ratio of 1.9 to 4.9 kb band, indicating a low level of 5′-B sites methylation, was high for adult DNA, while the ratio of 4.9 to 1.9 kb band was high in fetal DNA. In agreement with this result, when both DNAs were digested with *Xba*I + *Bss*HII, the 2.6-kb band was expressed more in adult than in fetal pancreas. The two *Xba*I sites exist on each side of the 5′- and 3′-B sites (one more, *Bss*HII site exists in second intron, plain "B" in Fig. 1A). Therefore, the 3.7 kb band resulting from *Xba*I + *Bss*HII digestion indicates the methylation of the 3′-B, but not 5′-B sites.

In adult rats, CCKAR mRNA has been found in the pancreas, but not in the liver, kidney or brain by Northern analysis (7, 8). To compare the status of DNA methylation of 5′-B sites for these tissues, genomic

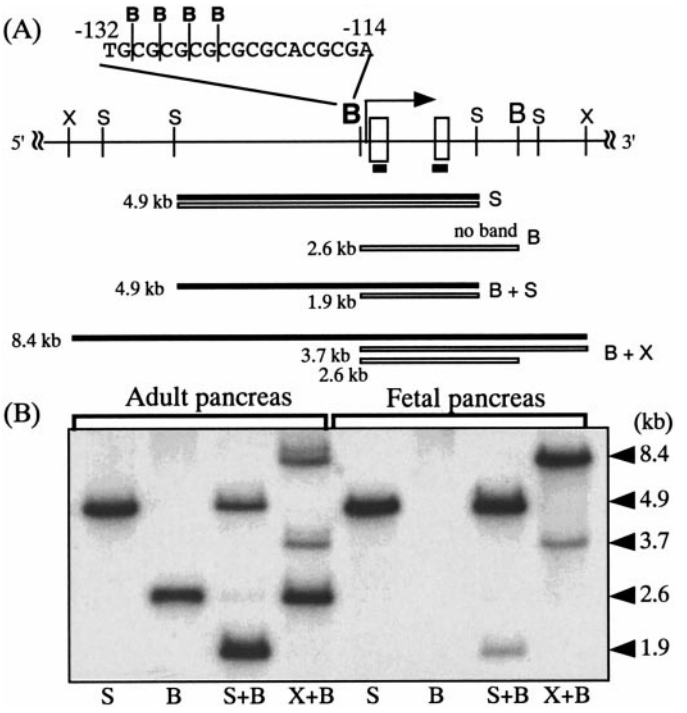

FIG. 1. Comparison of the methylation status of the CCKAR gene promoter in the adult and fetal rat pancreas. (A) Restriction map of the rat CCKAR gene DNA and sizes of the predicted digestion products. Open boxes on the map represent exon 1 and 2. Solid lines below the map indicate the 0.4 kb fragment from the CCKAR cDNA used as probe. The major transcription start site (+1) are shown by an arrow. There are two minor transcription starts at the position of −15 and −14 (9). Closed or open bars indicate predicted sizes for methylation or no methylation at the *Bss*HII sites (bold "B" between −132 and −114), respectively. The bold and plain B designate 5′- and 3′-B sites, respectively. Abbreviations for restriction enzymes: S; *Sac*I, B; *Bss*HII and X; *Xba*I. (B) Southern blot analysis of genomic DNA (30 μg) from adult (8-week-old female rats) and fetal (littermates of 19 days of gestation) rat pancreas. Arrow heads indicate some of the supplementary bands generated by each enzyme digestion. The DNA was digested with S, B, S + B and X + B. The digests were fractionated by electrophoresis in 1.0% agarose gel, transferred to a nylon membrane, and hybridized to CCKAR-specific cDNA probe.

DNA from various tissues of female rats was digested with *Bss*HII and *Sac*I + *Bss*HII, and a similar analysis was done. As shown in Fig. 2, the patterns for digested DNA in pancreas apparently differed from those in other tissues.

The pancreas is composed of about 90% acinar cells and 10% ductal and islet cells (11). The expression of CCKAR mRNA is found in acinar cell lines, AR42J cells (7), but be not examined in duct cells. We examined correlation between CCKAR expression and methylation status in the pancreatic AR42J (acinar) and ARIP (duct) cell lines. The CCKAR mRNA found in AR42J cells, but not in ARIP cells (Fig. 3A). The digestion of AR42J cell DNA revealed an intact 2.6 (*Bss*HII) and 1.9 kb (*Sac*I + *Bss*HII) fragments, on the other hand, the ARIP cells showed only 4.9 kb (*Sac*I +

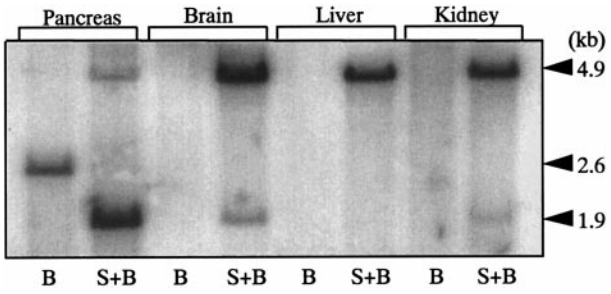

FIG. 2. Comparison of the methylation status of the CCKAR gene promoter in the pancreas, brain, liver and kidney. Southern blot analysis of genomic DNA (30 μg) from each tissue of female rats (5-week-old) was performed as described in the legend to Fig. 1. The DNA was digested with B or S + B.

*Bss*HII) fragments and no *Bss*HII digestion fragment (Fig. 3B).

In pancreas, the expression of CCKAR mRNA in newborn rats has not been reported previously. To examine developmental change of CCKAR mRNA levels in newborn rat pancreas, Northern blot analysis was performed with total RNA of pancreas at day 3, 7, 14, 21, and 35 after birth (Fig. 4). The expression increased markedly in 21 days after birth (Fig. 4A). CCKAR mRNA was quantitated as the ratio to β-actin mRNA on each day after birth (Fig. 4B). Taking the value for 35-day-old mature rats 100%, the expression at 14 and 21 days was 15 and 153%, respectively.

To examine the correlation between the expression and methylation, the DNA methylation status of 5′-B sites in the pancreas was determined in rats of various ages (Fig. 5A). Demethylation in the 5′-B sites is indicated by an increase in the intensity of the 2.6 (with *Bss*HII) or 1.9-kb band (with *Sac*I + *Bss*HII). In the

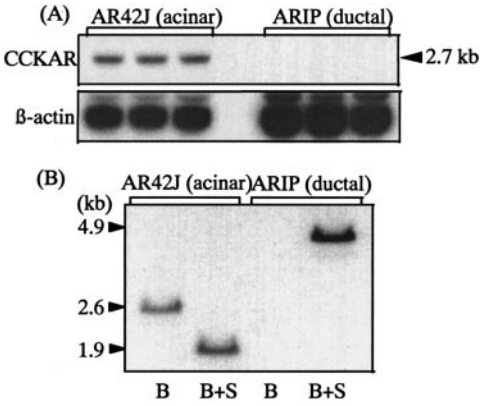

FIG. 3. Comparison of CCKAR mRNA expression (A) and methylation status (B) in the AR42J or ARIP cell lines. (A) Northern blot analysis of total RNA (20 μg) from AR42J and ARIP. The probe was described in the legend to Fig. 1. The 2.7-kb band indicates rat CCKAR mRNA. (B) Southern blot analysis of genomic DNA (30 μg) from AR42J and ARIP. Southern blot analysis was performed as described in the legend to Fig. 1.

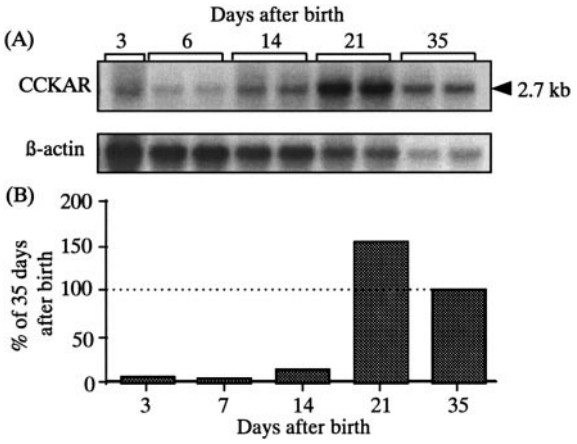

FIG. 4. Developmental change in CCKAR gene expression in the rat pancreas. (A) Northern blot analysis of total RNA (20 μg) from newborn rat pancreas. Total RNA was prepared from pancreas at days 3, 7, 14, 21, and 35 after birth. The 2.7-kb band indicates rat CCKAR mRNA. (B) Relative CCKAR mRNA levels in pancreas at each age. CCKAR mRNA was quantitated as the ratio to β-actin mRNA for each day after birth. Each bar expresses a percentage of the value 35 days after birth.

21-day-old rats, the 2.6 kb band is recognizable and the intensity of the 1.9-kb band is stronger than that of the 4.9-kb band. The extent of demethylation was evaluated as the ratio of *Bss*HII-digested DNA (2.6 kb) to *Sac*I + *Bss*HII-digested DNAs (1.9 + 4.9 kb). Taking the value for 35-day-old mature rats 100%, the expres-

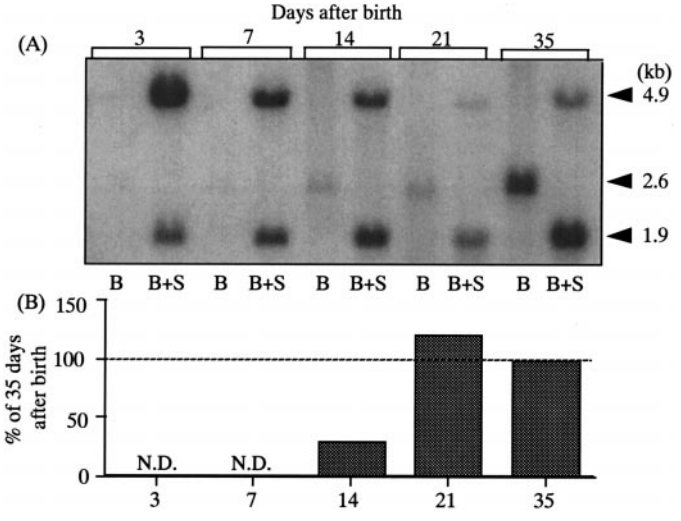

FIG. 5. Pancreas-specific DNA demethylation of the CCKAR gene promoter in developing rats. (A) Southern blot analysis of pancreas genomic DNA (30 μg) during development. Genomic DNA was prepared from 2 to 10 rat pancreas at each age, and digested with B and S + B. (B) Relative level of demethylated DNA (1.9 kb, *Bss*HII digests) in pancreas of each age. Hybridization signals (2.6 kb, *Bss*HII digests) of CCKAR genomic DNA were quantitated as the ratio to *Bss*HII–*Sac*I digest signals for each day after birth. Each bar expresses a percentage of the value 35 days after birth. N.D., not detectable.

sion in 14- and 21 day-old rats was 28 and 120%, respectively (Fig. 5B). Similarly, the status of methylation of 5′-B sites was also determined at each age for rat brain, liver and kidney. In contrast to pancreas, there was no developmental change in the status of methylation (data not shown).

DISCUSSION

In the present study, we demonstrated that the CCKAR gene is methylated in the fetal pancreas in which CCKAR is not expressed, but less methylated in the adult pancreas in which it is expressed (Fig. 1B). In addition, the adult pancreas was much less methylated than non-expressing tissues (Fig. 2). These results reveal an inverse correlation between methylation of 5′-B sites and expression of the CCKAR gene, and suggest that DNA methylation may contribute to tissue-specific and postnatal-dependent expression of the CCKAR gene.

The 5′-B sites in adult pancreas remained partially methylated (*Sac*I + *Bss*HII in Figs. 1B and 2). The methylation was not observed in the acinar AR42J cell DNA but observed in the ductal ARIP cell DNA (Fig. 3B). Previously, in binding experiment using labeled CCK, it suggested that rat pancreatic ductal cells has no CCKAR (12). This supports our result (Fig. 3A). It appears that partially DNA methylation in the adult pancreas came in part from ductal and/or islet-cells.

It is of interest that the DNA demethylation of 5′-B sites gives rise to specific and postnatal-dependent expression in pancreas (Figs. 4 and 5). In pancreas, a trace of CCKAR mRNA was detected at 3 days after birth, and the level of the mRNA rose rapidly up to day 21. Coinciding with this increase of mRNA, was a rose in the level of demethylation until 21 days of age. This expression of CCKAR mRNA in the newborn rats supports that the binding capacity of receptor for labeled CCK increases rapidly in 21 days rat pancreas, with no change in receptor affinity during development (13). In addition, we found that the postnatal-demethylation in the 5′-B sites is not observed in liver, kidney or brain, only in pancreas. In postnatal 3 and 7 days, the 1.9 kb band was observed but 2.6 kb band was not. These seem to come from postnatal difference of methylation status in 5′- and 3′-B sites (Fig. 1A). The 2.6-kb band is a digest from both 5′- and 3′-B sites, and the 1.9-kb band is a digest from 5′-B sites. The demethylation of 3′-B site is likely to correlate with CCKAR expression during postnatal development. In an early report, Baik *et al.* (14) showed that during rat kidney development, a progressive demethylation occurs in the γ-glutamyl transpeptidase gene. Their and our results suggest that the selective appearance of developmental demethylation is gene and tissue specific. Although it is not clear whether other genes in the pancreas are demethylated, we conclude that the postnatal-demethylation may be important in the pancreatic expression of the CCKAR gene. These findings would account for the postnatal-dependent expression of the CCKAR gene in pancreas.

Several mechanisms of DNA demethylation have been proposed for mammals. For instance, the transcription factor Sp1 is implicated in the generation and maintenance of unmethylated DNA, in that Sp1 binding enhances demethylation to prevent methylation at this site (15). Recently, the cDNA encoding DNA demethylase was cloned from human cells. When the cDNA was transiently transfected into human cells, the demethylase activity was revealed (16, 17). In the CCKAR gene, it is not clear how demethylation occurs postnatally in pancreas. Further experiments will be required to demonstrate the mechanism of tissue-specific demethylation in detail.

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REFERENCES

1. Wank, S. A. (1995) *Am. J. Physiol.*, G628–G646.
2. Chu, M., Rehfeld, J. F., and Borch, K. (1997) *Carcinogenesis* **18,** 315–320.
3. Nakano, S., Kihara, Y., and Otsuki, M. (1998) *Pancreas* **16,** 169–175.
4. Wang, Y., Naruse, S., Kitagawa, M., Ishiguro, H., Nakae, Y., Yoshikawa, T., and Hayakawa, T. (1998) *Pancreas* **17,** 289–294.
5. Funakoshi, A., Miyasaka, K., Shinozaki, H., Masuda, M., Kawanami, T., Takata, Y., and Kono, A. (1995) *Biochem. Biophys. Res. Commun.* **210,** 787–796.
6. Monstein, H. J., Nylander, A. G., Salehi, A., Chen, D., Lundquist, I., and Hakanson, R. (1996) *Scand J. Gastroenterol.* **31,** 383–390.
7. Wank, S. A., Harkins, R., Jensen, R. T., Shapira, H., de, W. A., and Slattery, T. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 3125–3129.
8. Wank, S. A., Pisegna, J. R., and de, W. A. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 8691–8695.
9. Takata, Y., Takiguchi, S., Funakoshi, A., and Kono, A. (1995) *Biochem. Biophys. Res. Commun.* **213,** 958–966.
10. Ramsahoye, B. H., Davies, C. S., and Mills, K. I. (1996) *Blood Rev.* **10,** 249–261.
11. Githens, S. (1988) *J. Pediatr. Gastroenterol. Nutr.* **7,** 486–506.
12. Smith, J. P., Yelamarty, R. V., Kramer, S. T., and Cheung, J. Y. (1993) *Am. J. Physiol.* **264,** G1177–G1183.
13. Hadjiivanova, C., Dufresne, M., Poirot, S., Sozzani, P., Vaysse, N., Moroder, L., and Fourmy, D. (1992) *Eur. J. Biochem.* **204,** 273–279.
14. Baik, J. H., Siegrist, S., Giuili, G., Lahuna, O., Bulle, F., and Guellaen, G. (1992) *Biochem. J.* **287**(Pt. 3), 691–694.
15. Silke, J., Rother, K. I., Georgiev, O., Schaffner, W., and Matsuo, K. (1995) *FEBS Lett.* **370,** 170–174.
16. Bhattacharya, S. K., Ramchandani, S., Cervoni, N., and Szyf, M. (1999) *Nature* **397,** 579–583.
17. Ramchandani, S., Bhattacharya, S. K., Cervoni, N., and Szyf, M. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 6107–6112.