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Transgenic Mice Overexpressing Cocaine-Amphetamine Regulated Transcript in the Brain and Spinal Cord

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ABSTRACT

Cocaine-amphetamine regulated transcript (CART), a satiety factor regulated by leptin, is associated with food intake and motor behavior. In knock out studies, Leu34Phe mutation of human CART gene resulted in obese phenotype but mice carrying a targeted deletion of the CART gene exhibited no dramatic increase of body weight on normal fat diet. To establish a new transgenic mouse model for determining the function of CART on feeding behavior *in vivo*, we constructed the fusion gene, CART gene under the control of neurofilament light chain promoter, which regulates gene expression at the stage of neuronal differentiation. Transgenic mice were generated by microinjection method and screened by PCR and Southern blot analyses. In these transgenic mice, overexpression of CART was detected by *in situ* hybridization in spinal cords and brains at 13.5 days post-coitum embryos. At six weeks of age, RT-PCR analysis showed that exogenous CART mRNA was expressed strongly in brains and spinal cords, but not much in other tissues. Our results suggest that these transgenic mice provide a new model to investigate the function of CART gene in neuronal network associated with feeding behavior.
(Key words : CART, NF-L Promoter, Transgenic, Aorexigenic, Feeding behavior)

Ⅰ. INTRODUCTION

Cocaine-amphetamine regulated transcript (CART) up-regulated with the acute administration of cocaine and amphetamine was initially identified by ploymerase chain reaction (PCR) differential display (Dauglass et al., 1995). Alternative poly(A^+) site utilization results in two transcript species that were approximately 700 or 900 nucleotides in length (Dauglass et al., 1995). CART contains three exons and 39 additional nucleotides, attributable to alternative splicing, at second exon in rat and mouse (Adams et al., 1999; Dauglass et al., 1995). In addition, CART peptide is divided differently by being processed in the periphery and in the central nervous system (Dauglass et al., 1996; Thim et al., 1999).

CART mRNA is expressed in hypothalamic regions thought to be important in the control of food intake including the zona incerta, the paraventricular nucleus of the hypothalamus, leteral hypothalamus area, dorsomedial hypothalamus and the arcuate nucleus (Arc) (Vrang et al., 1999; Kristen-

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sen et al., 1998). CART-immunoreactive localization was densely detected in hypothalamic area as well as other tissue such as superficial layers of rat dorsal horn, pituitary and adrenal gland (Couceyro et al., 1997; Koylu et al., 1997).

It was founded that CART is a mediator of anorexigenic signal from adipose tissue and cholecystoknin-induced satiety. Hypothalamic CART mRNA is decreased in leptin deficient *ob/ob* mice and increased by leptin treatment in *ob/ob* mice and starved rats (Kristensen et al., 1998). CART mRNA expression in rat vagus nerve and nodose ganglion is markedly attenuated after vagotomy, but no modulation is observed after food restriction (Broberger et al., 1999).

CART injected into discrete region shows different physiological roles *in vivo*. An anorexigenic signal of CART was supported by the result that intracerebroventricular (i.c.v.) administration of recombinant CART (55-102) inhibited feeding both in non-fasted rats and in neuropeptide Y (NPY) injected rats (Kristensen et al., 1998). It was reported that the rats i.c.v. injected into forth ventricle with CART (55～102) showed behavioral change that resembled the typical behavioral alterations found in the mice carrying disorders in the brain serotonergic system (Aja et al., 2001). However, CART (55-102) injected intranuclearly into hypothalamic area caused significant increase of food intake (Abbott et al., 2001) and this peptide injected intrathecally caused a dose-dependent and significant decrease of paw withdrawal latency (Ohsawa et al., 2000).

Leu34Phe mutation of human CART gene resulted in obese phenotype and reduced resting energy expenditure. Mice carrying a targeted deletion of the CART gene exhibited obesity phenotype on high caloric diet, but no dramatic increase of body weight in normal diet (del Giudice et al., 2001; Asnicar et al., 2001). Therefore, to define further the physiological role of CART *in vivo*, we generated transgnic mice overexpressing mouse CART in brain and spinal cord under the control of neurofilament light chain (NF-L) promoter, which regulates expression both neuron and muscle in developmental stage (Yaworsky et al., 1997).

Ⅱ. MATERIALS AND METHODS

1. DNA Constructs

Genomic DNA was extracted from tail samples of B6CBAF1 (C57BL/6J×CBA/J)F1 mice by phenol extraction methods (Hogan et al., 1986). Then, to clone CART and NF-L promoter gene, PCR amplification was performed by using primers for CART (5'-AGCAGCGAGGAGGTCCAGAA-3' and 5'-ACCA ACACCATTCGAGGCAT-3') and NF-L promoter (5'-TTGCAGCAGTCTCTC TCTCTCTC-TC-3' and 5'-TAGCCGAACGAACTCATGGTG-3'). Thermal cycling profiles were as follows: denaturation at 94℃ for 30 sec, annealing at 55℃ for 30 sec, and extension step at 72℃ for 120 sec for 35 cycles. All primers for PCR amplification were designed according to the published mouse CART (accession No. AF148071) and NF-L promoter (accession No. U80021) sequence available from Genbank (Adams et al., 1999; Yaworsky et al., 1997). To confirm exact sequence, PCR products were subcloned into the T overhang site of pCR® 2.1-TOPO vector (Invitrogen, CA, USA). DNA sequencing was performed by Macrogen Co., Korea.

To detect NF-L promoter activity, NF-L-pEGFP vector was constructed by ligating pEGFP-N1 vector (Clontech, CA, USA) with NF-L promoter sequence, which was deleted with Kpn Ⅰ and Xma Ⅰ to induce neuron specific expression (Yaworsky et al., 1997). In order to express CART in neurons, the fusion gene comprising of both NF-L promoter and CART gene was constructed by ligating NF-L-pEGFP vector with CART gene digested with

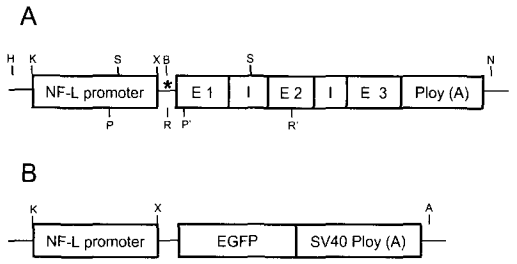

Fig. 1. Schematic diagrams and partial restriction maps of DNA constructs. A) neurofilament light chain (NF-L) promoter/CART, B) NF-L promoter/EGFP, Asterisk indicates sequence containing both 5'-noncoding region of light chain gene and a part of inserted pCR 2.1-TOPO vector, E; exon, I; intron, P and P'; primer for PCR, R and R'; primer for RT-PCR, H; Hind Ⅲ, K; Kpn Ⅰ, X; Xma Ⅰ, S; Sac Ⅱ, B; Bam H Ⅰ, N; Not Ⅰ, A; Afl Ⅱ.

Bam H Ⅰ and Not Ⅰ (Fig. 1).

2. Generation of Transgenic Mice

Mice of B6CBAF1 (C57BL/6J×CBA/J)F1 and ICR strains were purchased from Daehanbiolink Co., Korea. Female mice were superovulated by an i.p. injection of 5 IU PMSG and followed by i.p. injection of hCG 48 hr later. Mice were killed between 18 and 20 hr after hCG injection by a cervical dislocation. The pronuclear-stage zygotes were collected and the cumulus cells were removed in M2 medium containing 300 μg/ml of hyaluronidase. The one-cell eggs were centrifuged at 14,000 x g for 10 min at 37℃ to reveal the normally obscure pronuclei. NF-L-CART vector was digested with Hind Ⅲ and Not Ⅰ for microinjection. Linear NF-L-CART fusion gene were dissolved respectively as a 10 μg/ml of concentration in 10 mM Tris (pH 7.4) and 1 mM EDTA buffer. Approximately 1 to 2 *pl* containing about 10 μg/ml of DNA solution was injected into the pronucleus of the one-cell embryos. Microinjection was performed at 400× with a Hoffman Modulation Contrast Nikon objective on a Nikon inverted microscope (Eclipse TE 300; Nikon Inst., Japan) fitted with a micromanipulator (Narishige Co., Japan) following the methods of Hogan et al., (1986).

3. PCR and RT-PCR Analyses

At one week age, tail biopsy was performed by clipping 0.5 cm from each pup at the time of weaning. Each genomic DNA was extracted from the tail samples by phenol extraction methods (Hogan et al., 1986). Then, PCR was performed by using primers (5'-ACTGACCAGCTCCTTCTCGT-3' and 5'-ATCGATCACAGTCTGCGCTA-3'). Primers were designed to detect the sequence containing both a part of NF-L promoter and genomic CART sequence. Thermal cycling profiles were as follows: denaturation at 94℃ for 30 sec, annealing at 65℃ for 30 sec, and extension step at 72℃ for 30 sec for 35 cycles.

Total RNA was extracted from NF-L-CART vector transfected HeLa cells and the various tissues in transgenic mice at 6 weeks of age by using Trizol (Gibco BRL, NY, USA). Contaminating DNA was removed by treating 1 μg total RNA with DNase Ⅰ. The first strand cDNA was synthesized from 1 μg total RNA with 200 U Superscript Ⅱ Reverse Transcriptase (Gibco BRL, NY, USA). The primers used for CART in transfected HeLa cells were the same for cloning of genomic CART gene. Thermal cycling profiles were as follows: denaturation at 94 ℃ for 30 sec, annealing at 65℃ for 30 sec, and extension step at 72℃ for 30 sec for 35 cycles. The primers were used for exogenous CART (5'-CCGTTCTTCTCTCTAGGTCC-3' and 5'-AGGACTTC TTGCAACGCTTC-3') and β-actin (5'-CAGAGCAAGAGAGGTATCCTGA-3' and 5'-CTCTAGAGCAACATAGCACAGC-3'). The primers for exogenous CART, which contains both 5'-noncoding region of

light chain gene and CART second exon were designed to detect two types of exogenous CART transcripts by alternative splicing. PCR amplification was performed to detect exogenous CART for 31 cycles. To detect β-actin as a control, PCR amplification was performed for 26 cycles. Thermal cycling profiles were as follows: denaturation at 94 ℃ for 30 sec, annealing at 60℃ for 30 sec and extension at 72℃ for 20 sec.

4. Southern Blot Analysis

Southern blot analysis of CART gene was performed to confirm transgene integration and copy number of transgene following the methods of Sambrook et al., (2001). DNA concentration was measured by the ratio of A_{260}/A_{280} with a spectrophotometer (Beckmann, CA, USA). DNA (10 μg per mouse) was digested with Sac Ⅱ, electrophoresed on 0.7% agarose gels, transferred to nylon membrane (Hybond N$^+$ Co., S&S, USA) by using a capillary membrane transfer method. A standard equivalent to 10, 50 and 100 copies of transgene was also electrophoresed. Transferred membranes were hybridized with in phosphate-SDS buffer to probe, which is the 1019 bp Kpn Ⅰ-Bam HⅠ fragment of the NF-L promoter labeled with P_{32}-dCTP by using rediprimeTM Ⅱ (Amersham Pharmacia Biotech, UK).

5. *In situ* Hybridization in 13.5 Days dpc Embryos

In situ hybridizations were performed as described previously (Jahng et al., 1998). Embryos at 13.5 days post-coitum (dpc) were isolated after natural mating between male founder transgenic mouse and female normal B6CBAF1 (C57BL/6J×CBA/J)F1 mice, washed in PBS, fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB) for 3 hr and transferred into 30% sucrose. Eighty-micron transverse sections were cut on freezing and sliding microtome. Sections were collected into 20 ml glass scintillation vials containing ice-cold 2×SSC (0.3 M NaCl, 0.03 M Na Citrate) for *in situ* hybridization. Sections were suspended in 1 ml of prehybridization buffer (50% formamide, 10 % dextran sulfate, 2×SSC, 1×Denhardt's solution, 50 mM DTT, and 0.5 mg/ml denatured salmon sperm DNA). After 2 hr prehybridization at 48℃, labeled denatured CART cDNA probe (1×10^7 CPM) was added to the vials, and hybridized overnight at the same temperature. The 606 bp fragment of CART cDNA cloned in our lab was labeled with S^{35}-dATP by the random-priming method (Boehringer Mannheim). Following hybridization, the sections were washed at 15 min intervals in decreasing concentrations of SSC (2×, 2×, 1×, 0.5×, 0.25×, 0.125×, 0.125×) at 48℃. The tissue sections were then mounted on gelatin-subbed slides, air-dried, and exposed to Kodak BioMax film (Eastman Kodak Co., NY, USA) at 4℃ during 48 hr.

Ⅲ. RESULTS

1. Construction of Expression Vector

The 1221 bp NF-L promoter and 1949 bp CART gene was cloned from B6CBAF1 (C57BL/6J×CBA/J)F1 mice genomic DNA. Our clone sequences resulted in some mutation in NF-L promoter (2 nucleotides deletion and 12 substitution in NF-L promoter 2) and in noncoding region of CART (2 deletion and 2 substitution) gene compared with previously published data (Yaworsky et al., 1997), but conserved sequence in rat and human was not changed (data not shown). NF-L-CART fusion gene comprising both the NF-L promoter 1015 bp fragment deleted with kpn Ⅰ and CART gene was constructed similar with NF-L-EGFP coding sequence (Fig. 1). The activity of NF-L promoter was observed by fluorescent image on HeLa cells by using

fluorescent microscope following transfection of NF-L-EGFP constructs in vitro. RT-PCR analysis showed that expression of CART mRNA was detected in HeLa cells transfected with NF-L -CART vector, but not detected in non-transfected HeLa cell.

2. Generation of Transgenic Mice

Twenty-four offsprings were obtained from 106 embryos transferred after microinjection of Hind Ⅲ -Not Ⅰ fragment of NF-L-CART (3 kb). Five of them were transgenic. Except one, Four of them transmitting NF-L-CART gene through their germline were determined by PCR (Table 1). Transgenic mice were confirmed by Southern blot analysis (Fig. 2). In densitometry analysis compared with a standard equivalent to 10, 50 and 100 copies of transgene, approximate copy numbers of NF-L-CA-RT transgenes were as follows: 10 copies in #1, 4; 15 copies in #2; 20 copies in #3. PCR results were identical to those of Southern blots, and so PCR was used to facilitate analysis of transgenic mice.

Table 1. Detection of transgenic offsprings by PCR and Southern blot analyses

No. of embryos transferred	No. of offsprings	No. of recipients	No. of transgenic mice
105	24	5	5 (21%)[a]

[a] No. of transgenic mice/ No. of embryos transferred ×100.

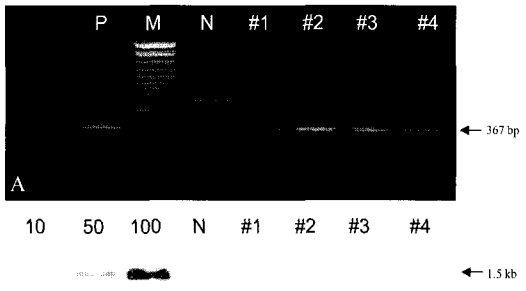

Fig. 2. Detection of transgenic mice. A) PCR anlaysis, B) Southern bolt analysis, 1, 10 and 100; copy numbers of transgene, P; Positive control, N; Negative control, M; 100 bp ladder, #1, #2, #3 and #4; transgenic mice.

3. Expression of Exogenous CART mRNA in Transgenic Mice

RT-PCR analysis showed both the 322 bp and the 283 bp transcripts of exogenous CART mRNA expression were detected from various tissues in transgenic mice (Fig. 3). Densitometry results indicated that CART mRNA was highly expressed by about 90% in brain and spinal cord compared with intestine, muscle, heart, liver, kidney and ovary in adult transgenic mice. However, exogenous CART was not detected in normal mouse brain. Results of *in situ* hybridization to investigate expression pattern of CART in 13.5 dpc embryo stage revealed

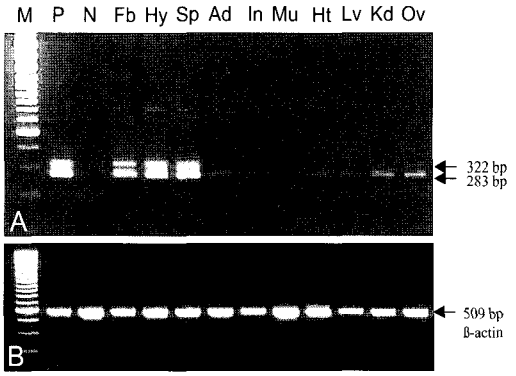

Fig. 3. Expression of mRNA in various tissue from #3 transgenic F1 mice by RT-PCR. A) exogenous CART mRNA, B) β-actin mRNA as control, P; positive control, N; negative control, Fb; fore brain, Hy; hypothalamus, Sp; spinal cord, Ad; adrenal gland, In; intestine, Mu; muscle, Ht; heart, Lv; liver, Kd; kidney, Ov; ovary.

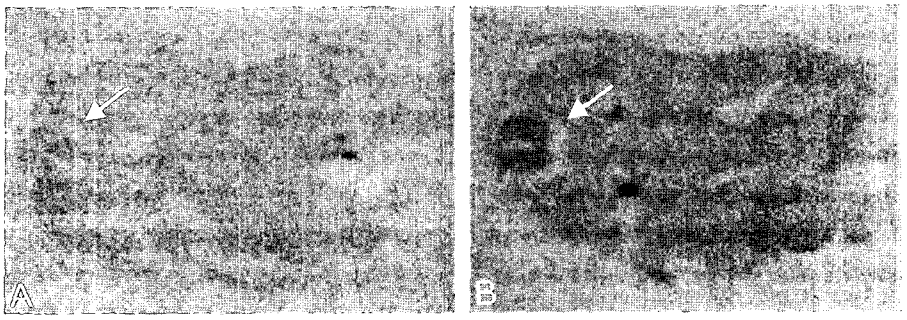

Fig. 4. CART mRNA expression in situ hybridization at 13.5 dpc embryo. A) normal embryo, B) #3 transgenic mice. The white arrow indicates CART overexpression in spinal cord.

that CART mRNA expression was dispersed through the whole brain in transgenic mice. CART mRNA expression at spinal cords in transgenic mice compared with normal mice showed a significant increase (Fig. 4).

Ⅳ. DISCUSSION

Localization of CART mRNA and immunoreactivity were detected in hypothalamus, pituitary and neuron cell by using *in situ* hybridization and immunohistochemistry. To investigate CART's role of in neuronal network associated with a feeding behavior, we generated transgenic mice overexpressing CART in brain and spinal cord. We cloned mouse CART and NF-L promoter sequence, constructed expression vectors comprising of EGFP or CART under control the of NF-L promoter and determined expression of DNA constructs *in vitro* by fluorescent microscopy and RT-PCR analysis following transfection into HeLa cells. These results confirmed NF-L promoter activity on transformed non-neuronal cell and CART gene expression under the control of NF-L promoter (Reeban et al., *1995*).

In our transgenic mice, CART mRNA expression was expressed in spinal cord at 13.5 dpc embryo stages and was expressed not so much through the whole brain in transgenic mice carrying exogenous CART gene. In adult, RT-PCR analysis showed both the 322 bp and the 283 bp transcripts of exogenous CART was highly expressed by about 90% in brain and spinal cord compared with various tissues. Both the 322 bp and 283 bp transcripts of exogenous CART gene showed that the transgenes might be acted exactly in transgenic mice and they were alternatively spliced such as endogenous CART gene. In previous report, CART mRNA expression was low during the neonatal period and increased to near adult levels after weaning (Ahima et al., 2000). However, In our results, CART expression was detected in 13.5 dpc transgenic embryos, which indicated expression of exogenous CART gene but not that of endogenous. *In situ* hybridization and RT-PCR analyses showed expression of exogenous CART gene was regulated by the NF-L promoter. It was reported that NF-L promoter fragment including −941 base pairs of 5'-flanking sequence contained a sufficient element for expression of transgene in neuronal and myogenic tissues because MyoD binding site at −928 of NF-L promoter may confer the muscle-specific promoter activity. NF-L promoter activity was week at neonatal *10 days and* not maintained in mature neuron (Yaworsky et al., 1997). However, this experiment showed NF-L activity was highly expressed by about 90% in barins compared with muscle and maintained at six weeks

of age. These results indicated the possible presence of an additional neuron-specific regulatory element in mouse CART gene and CART might not affect the neuron development and the physiological regulation at an early developmental stage.

Then, we hypothesized that increase of CART expression in hypothalamus and spinal cord might induce the reduction of food intake. However, transgenic mice showed no significant differences in behavioral phenotype. Orexic molecules such as NPY and agouti-gene related proteins were co-localized in Arc neurons (Broberger et al., 1998; Hahn et al., 1998) and anorexic molecules such as CART and pro-opiomelanocortin were co-localized in Arc neurons (Elias et al., 1998). In the Arc, the secretion of these peptides regulating ingestive behavior was regulated by leptin levels (Stephens et al., 1995; Ahima et al., 1999; Ebihara et al., 1999; Thornton et al., 1997; Kristensen et al., 1998). In our transgenic mice, overexpression of CART mRNA through the whole brain and spinal cord may be compensated by orexic molecules co-localized in various brain area or other regulation mechanism. Although overexpression of CART in brain and spinal cord of transgenic mice showed no effect on behavioral phenotype, it is suggested that transgenic mouse model can be useful in study the function and molecular correlation within neuronal network in feeding behavior.

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요 약

뇌와 척수에서 Cocaine-Amphetamine Regulated Transcript를 과발현하는 형질전환 생쥐

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CART는 leptin에 의해 조절되는 포식인자이며 섭식과 운동 습성에 관계된 것으로 알려져 있다. 사람의 CART Leu34Phe 돌연변이는 비만의 표현형을 나타내었지만, 생쥐의 CART 돌연변이는 일반사료의 섭취 후 급격한 체중증가를 나타내지는 않았다. 생체 내 신경세포에서 CART의 역할을 확인하기 위한 새로운 형질전환 모델을 확립하고자 분화하는 신경세포의 유전자 발현을 조절하는 NF-L promoter와 CART의 재조합 발현 벡터를 구축하였다. 형질전환 생쥐는 유전자 미세 주입법에 의하여 생산되었으며, PCR과 Southern blot의 방법으로 확인하였다. 이러한 형질전환 생쥐에서 CART의 과 발현을 수정 후 13.5일째 초기 배아와 생후 6주째 형질전환 생쥐의 뇌와 척수에서 확인하였다. 본 연구의 결과는 섭식 관련 유전자들이 상호 연관된 섭식행동에서 CART의 역할을 연구하는데 모델 동물로써 이용할 수 있을 것으로 사료된다.

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